

When to blow the whistle

A failed prosecution in the British courts has caused a political sensation; the underlying question is when and how professional people should disagree with their employers.

ALL British readers of this journal are bound by a piece of legislation called the Official Secrets Act of 1911, consisting of two separate interdictions. One is a requirement that British nationals should not communicate information affecting the interests of the state to representatives of foreign governments, which is sensible enough. The second is an interdiction of the transfer of information touching the "interests of the state" to anybody not authorized to receive it, which is a hopeless piece of legal drafting, to say the best. This law applies to all British nationals but many have been required to go further than this, and to sign a piece of paper on which the interdictions of the act are printed. The purpose of "signing the Official Secrets Act" as this process is described is to remind people that the act exists and to warn them of the huge scope of its interdictions, especially the second. At a guess, half of *Nature's* British readers may be thus compromised, either because they work for the government or have, from time to time, given it advice.

The chances are only meagre that these repressive provisions will be banished by the failure last week of the British government's prosecution under the Official Secrets Act of a civil servant at the Ministry of Defence. The circumstances are that a Mr Clive Ponting leaked information to a British Member of Parliament about an incident during the Falklands war, the sinking of an Argentinian vessel with the loss of nearly 1,000 lives. Mr Ponting acknowledged during his trial that he had indeed passed on the information; his defence was that his action was justified because he believed the government was seeking to mislead the House of Commons. The willingness of the trial jury to accept this argument has inevitably caused a great hullabaloo, so much so that one British newspaper says that British politics is "suddenly interesting again".

The issue that most immediately concerns politicians is whether the British government sought to cover up a shabby incident (and, if so, which heads should roll) and whether political advantage can be quarried from the mess. The danger, in all this fuss, is that people will forget that Mr Ponting's problem was (by his account) one of conscience. He believed that his employer, a government department, was acting in a manner inconsistent with its public responsibilities. All professional people working for employers, whether governments or not, are at risk of running into such difficulties, which is why the Ponting case concerns not just the half of *Nature's* British readership that happens to have signed the Official Secret Act but everybody, everywhere.

Even governments acknowledge that there are circumstances when professional people must rebel if asked to violate their public responsibilities. This is the spirit in which ex-officials of the Nazi government found, at the Nuremberg trials of the 1940s, that it was not a sufficient defence to say that they had been carrying out instructions. The problems of conscience that now arise are less stark than those that should have haunted the people convicted at Nuremberg, but in practice that is a complicating circumstance. It may be harder for professional people to judge when the duty is expected by their employers is incompatible with their general responsibility, while there may be a great many of these occasions to contend with. Where should the line be drawn between duty to employers and to a wider public?

That an employee has responsibilities towards his employer is self-evident. Those working for commercial organizations have

a duty not to damage their employers' commercial interests, either by bad-mouthing the organization or by passing on confidential information (financial or technical) to others. So much is widely understood and accepted, and sometimes even enshrined in law. Within such a framework, problems of conscience that cannot be tidily dealt with may easily arise. People working in defence research establishments, for example, may be conscientiously opposed to the use of nuclear weapons, poison gas or some other weapon system, and may thus be placed in an impossible position if they are asked to work on the development of such devices. Or, in a commercial organization, people may resent their employer's diversification into the manufacture of cigarettes or of wire-tapping equipment or the provision of a polygraph service. Ordinarily, people have little hope of redress in such circumstances.

If a government is constitutionally and legally within its rights in making novel weapons, however distasteful, or if a company remains within the law by taking on even anti-social or foolish business, there is little that an employee can do but find a more congenial employer. It is even proper that an employer embarked on such a course should be entitled to require that employees should not use external channels to oppose whatever decisions may have been reached, which is why there can be only a modest fund of public sympathy for, say, temperance campaigners who earn their livings from distilleries, or anti-nuclear campaigners working for electricity utilities. There is a case for asking that professional societies should do more to help such people to find other jobs, but otherwise there is little that can be done.

Mr Ponting's problem is more subtle, and probably more typical of the problems of conscience that most commonly occur. While not dissenting from the decision that the Argentinian vessel should be sunk, Mr Ponting (according to his evidence in the Central Criminal Court) considered that his employer was planning a misleading explanation of the circumstances flatly in conflict with the constitutional convention (in Britain, nothing is written) that the House of Commons should always be told the truth. The circumstances are exactly comparable with those in which, say, a technical organization may suppress information about the safety of its products to the ultimate users, or which may plan, in the conduct of its operations, to violate the law.

When and how should people in such a position blow the whistle? And what protection can they then be given? At one level, this is almost an administrative matter. Both government departments and commercial organizations need to equip themselves with mechanisms by which those who dissent from planned courses of action can say so, and in such a manner that they can be heard by those ultimately responsible with such clarity that the organization is in no doubt of the charges being made against it. The obvious difficulty is that even whistle-blowers who have correctly anticipated misdemeanours by their organizations do not endear themselves to their bosses. The fate of complainants is more often unpopularity. Promotion will rarely be thrust upon them. Yet employers as well as employees are damaged in these ways.

In everybody's interest, there is therefore a need for some mechanism by which legitimate professional dissent can surface within all organizations without the personal interests of the whistle-blowers being damaged. This is a field to which



professional associations and societies should pay more attention, perhaps following the physicians in their largely successful insistence that their responsibility to their patients takes precedence over that to their employers. There is no reason why, if organizations will not take sensible steps in this direction, legislation should not be used to compel seemly practice. Whistle-blowers who have failed to win satisfaction within their organization might even be protected if they make their complaints public. □

Embryos untouched

The British government must act quickly if it is not to lose the chance to regulate research.

AS expected, the British government is now in a fix over its plans to regulate research in human embryology. The danger that delay in following the report of the Warnock Committee last July with legislation would invite some lone Member of Parliament to take pre-emptive action has now materialized (see p.618). Mr Enoch Powell's bill is unlikely ever to become part of British law, given parliamentary procedures which, for practical purposes, will require either that the government should give the bill a helping hand or that the four private members ahead of Mr Powell in the queue should be willing to withdraw. Yet the size of the vote, and the tenor of the declarations in favour of the bill's proposal that embryos should never be subjects of research, will be an embarrassment for the government, which has still to decide in what form it will bring in its own legislation. The most the Minister for Health, Mr Kenneth Clarke, could promise last week was that there would be a set of proposals "within the lifetime of this Parliament", which could be three years hence.

The issues are complicated, but can they be so difficult? The most serious problem with which the government has to contend is the emotive character of public discussion, well illustrated by last week's debate, but also by Mr Powell's frank (and characteristically lucid) declaration that his opposition to research with human embryos stems from his "sense of revulsion, deep and distinctive, to the proposition that a thing, however it may be defined, of which the sole purpose is that it may be a human life, should be subjected to experiment to its destruction for the purpose of the acquisition of knowledge". Logically, Mr Powell went on to acknowledge that it is irrelevant to this position that potentially valuable research may never be undertaken.

For Mr Powell and many others, the issue is clear: a fertilized ovum is a human being and there is nothing more to be said. But is that indeed the case? A one-cell embryo can become a living person only if implanted in a uterus. No other means of converting one-cell embryos into people has been found. A project for designing artificial systems for maturation would be a daunting task, given the delicacy of the homeostatic systems that are involved, most of which are still unknown. Moreover, the intervention of a real uterus and the adult woman to whom it belongs is more than a mere source of nourishment; it is a means through which an embryo wins the emotional and physical security of its parents for much longer than the period of gestation. The Warnock Committee prudently dodged the over-simple question "when does life begin?", but who can object to the proposition that there can be no such thing as a living being without implantation?

That conclusion is not however a licence for unrestricted research with unimplanted embryos. Whatever may be the potential interest of investigations in this field, Mr Powell's instinctive revulsion is too widely shared to be ignored. That is why it is generally agreed that research must at least be regulated, preferably by means of the open decisions of a committee not dominated by researchers. In the absence of legislation, anything is possible and will luridly be supposed to be taking place. So why does not Mr Clarke take a leaf from Mr Powell's book and bring in a short bill of his own to set up a regulatory committee, to require that operations for the fertilization of human ova should be licensed, that those concerned should account for all

such embryos and also be required to seek approval in advance for all procedures other than the use of embryos in *in vitro* fertilization? The result would be acceptable to researchers, and would allow some research to proceed. It should also be acceptable to Mr Powell and his supporters, being an improvement on the present uncertainty. If it were as open as it should be, the procedure would also quickly nail the widespread supposition that laboratory people are just bursting to fashion Aldous Huxley's *Brave New World*. And Mr Clarke would then have time in which to deal properly with the other conundrums on the Warnock agenda. □

Reactors for trade

The plan to make the UK AEA function commercially hazards the independence of research.

ONCE upon a time, some thirty years ago, the British government created the UK Atomic Energy Authority in the belief that only a substantially independent public corporation could fully exploit the civil benefits of nuclear energy, previously a military business. For much of the early period of its existence, when most universities lived on shoestrings, the authority was one of the chief agents of research and an important source of technical innovation in Britain. But over the years, its pride has been dented in several ways. Thermonuclear power has proved less accessible than it seemed in 1958, thermal reactors went through a bad patch and the original hope that most British electricity would by now be generated in a careful mix of fast and thermal reactors has been put off for at least another thirty years. Then, last week, there came the final humiliation, for the authority is to be converted into what the British Treasury calls a "trading fund", organized as if it were a commercial entity which nevertheless will depend on the government.

This decision, announced by the Department of Energy on 11 February, is consistent with present British fashion of making as many government functions as possible into private entities standing on their own feet. Fair play, it is also true that much has changed since the authority came into being. In the past few years, the authority has hived off (as separate public companies) its interests in both fuel-processing and isotope applications (as British Nuclear Fuels and Amersham International respectively). The novelty of what is now proposed is that an entity which is essentially a research organization should be expected to run on commercial lines (beginning on 1 April 1986). The attraction of the new scheme is partly administrative tidiness and partly the hard calculation that an organization whose research is carried out for the nuclear power industry should win its support from its customers, the electricity undertakings. The first version of the Department of Energy's internal report on the subject put the issue in these stark terms. The version eventually published in abbreviated form last October went some way to recognize that a national nuclear industry cannot politically be entrusted with its own research on the safety of reactors, while even the conduct of long-term research (fast reactors, fusion, fission physics) cannot be fairly lumped as a charge on electricity consumers. Mercifully, these points seem now to have been taken.

For the time being, everybody seems mightily relieved. The British Department of Energy will continue to pay the authority for the cost of fast reactor research, now part of a joint European programme. The electricity utilities will pay for research and development directly related to reactor systems, but the government will foot the bill for attendant safety research. Research establishments such as Harwell, which has fifteen years of success in recruiting outside research contracts, will be expected to stand even more firmly on their own feet. But there will be a substantial part of the authority's present budget, concerned with basic research, that cannot be fairly charged against research contracts. Especially because most of the likely research customers are themselves monopolies, there are strong reasons why the government should match its wish to see an independent research capability with a determination to foot the bill. □

Space telescope

Hubble's terrestrial tail threatens to wag dog

Washington

WHEN the National Aeronautics and Space Administration (NASA) decided to establish an institute to manage scientific operations of the Hubble Space Telescope, it planned a staff of 100 and a budget of \$24 million over five years. But since the institute was established at Johns Hopkins University in 1981, NASA has seen the institute grow to a staff of more than 200, and, with the launch still at least 18 months away, an annual budget of \$12.8 million. Astronomers and NASA officials are now seriously concerned that costs for the project are out of control, and that other worthwhile projects, such as the theoretical astrophysics programme (now running at \$1 million a year) and the \$50 million-a-year Explorer satellite programme, are being squeezed out.

Total operating costs of the space telescope, including development of second-generation instruments and the operation of the Space Telescope Science Institute (STSI), are estimated to reach \$150 million a year, almost one quarter of NASA's physics and astronomy budget. This is on top of the \$1,400 million it has cost to develop the telescope.

Although some of the cost overruns have been the result of unforeseen technical complications in construction and in the necessary ground operations, the astronomy community has begun to look with growing apprehension at the expansion of the institute. The institute's budget request for fiscal year 1986 exceeds \$16 million; by 1989 the figure will be at least \$21 million, without allowing for functions such as data archives and distribution and telecommunications, which have still not been agreed upon. Frank Carr, NASA's deputy director of flight projects for the space telescope, has indicated that reserve funds have already been tapped to supplement this year's appropriation; budget negotiations with STSI have been "a struggle on both sides". The staff is also still growing, and is expected to reach between 250 and 270 by the time the telescope is in full operation; the institute's director, Riccardo Giacconi, is said to want a staff of over 400.

The rapid growth of the institute has led to some drastic measures to find extra office space.

One interpretation often heard in the astronomical community is that Giacconi is engaging in some not-too-subtle empire building, hoping to position the institute to assume scientific responsibility for future satellite observatories. Many of the facilities that would be needed for such future projects as the Advanced X-Ray

Astrophysics Facility (AXAF) are already in place at Giacconi's institute. AXAF would certainly be a desirable catch for the institute, some of whose staff, including Giacconi, have expertise in X-ray astronomy. The space telescope is scheduled to operate for only 15 years; more work at STSI would ensure continued employment for the institute's younger scientists, many of whom are untenured. A spokesman for the institute said Giacconi had no comment to make about the STSI budget or possible management of AXAF.

NASA top brass are thought to be set against allocating future missions to STSI, however. Some astronomers also have reservations: in congressional testimony last year, Robert Bless of the University of Wisconsin said it looked as if too much power was concentrated in one institution; Lyman Spitzer of Princeton University said that problems of communication are exacerbated in a large organization. Spitzer

hoped that if NASA adopts the institute model for managing future satellite observatories they would be kept separate to avoid creating "one large, ponderous, possibly unwieldy organization".

NASA, apparently as part of an effort to draw the line at further expansion of STSI, has commissioned a comprehensive external review of the institute's role from the National Academy of Sciences' committee on space astronomy and astrophysics. That study, which will be completed in April, is headed by William Gordon of Rice University. Gordon said the question of whether STSI should manage future observatories had been raised before his committee but that it would be premature for him to address the question until more experience had accumulated with STSI, which is a novel experiment for NASA. Questions about the space telescope operating budget have also been raised in an advisory panel headed by Jeffrey Linsky of the University of Colorado. Meanwhile, NASA has asked scientists at Goddard Space Flight Center to examine how expertise and facilities developed for the space telescope could best be applied to future satellite observatories by exploiting common functions.

Tim Beardsley

Japanese cadaver organs

Tsukuba group murder charge?

Tokyo

CONTROVERSY in Japan over the use of brain death (the absence of brain electrical activity) as a criterion of death is being heightened by a new legal challenge. Last week a group composed largely of Tokyo University Hospital doctors asked the Public Prosecutor's Office to charge a Tsukuba University group of three doctors with murder for their part in an operation performed last year.

In September, the Tsukuba group had operated on a 43-year-old woman who had collapsed from a stroke. All signs of brain electrical activity had ceased when the decision was made by the three doctors, Yoji Iwasaki, Katashi Fukao and Tadao Nose, to remove kidneys, pancreas and liver from the woman for a transplant operation. At the time, the woman's heart was still beating. It was the first time that such an operation had been performed in Japan.

Now, the 17-member Patients Rights Study Group has filed a complaint with the prosecutor's office on the grounds that, because the heart was beating, the patient was alive and the taking of organs thus tantamount to murder.

The case strongly recalls that of Toshiro Wada, who performed Japan's first heart transplant operation at Sapporo Medical College, Hokkaido in 1968. He, too, had taken brain death as the criterion of death before removing the heart of a drowned man for transplantation. A similar murder complaint was made by a volunteer group

but ultimately the case was dropped.

The furore unleashed at the time was, however, sufficient to prevent anyone attempting another heart transplant. It was only this year that the Ministry of Health and Welfare once more initiated a programme to build a consensus for public acceptance of brain death that would allow heart transplants to resume after a 17-year lapse (see *Nature* 313, 338; 1985). The new legal action is likely to bring matters to a head much more quickly than expected.

Reaction to the indictment from the medical profession has been mixed. Some have expressed regret that the Tsukuba operation was performed before attempts to have brain death accepted by public and doctors had made much progress. There is a fear that the public will now be led into thinking that the use of a brain death criterion will give doctors a free hand to transplant organs at will. On the other hand, there are those who think the case will actually hasten the preparation of new legislation aimed at making brain death the legal criterion of death.

Soon after the announcement of the indictment, a group of Diet members, drawn from all political parties, set up a Life Ethics Problem Study Parliamentarians League. The league contains lawyers, doctors and government officials as well as Diet members and hopes speedily to ascertain whether it is necessary to prepare a new law concerning the legal criterion of death.

Alun Anderson

Star wars

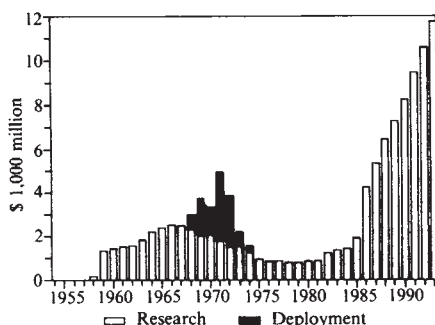
True cost still growing

Washington

INITIAL research and development on the Star Wars ballistic missile defence programme will probably cost more than \$70,000 million, according to a coalition of arms control groups opposed to the programme. The group, called the National Campaign to Save the ABM [Anti-ballistic Missile] Treaty, said last week that the usual description of the Strategic Defense Initiative (SDI) as a five-year, \$26,000-million research project is merely an artefact of the Pentagon's five-year planning window, and does not reflect the true extent of the effort.

With the release of the proposed 1986 budget, the programme has already become a six-year, \$33,000-million research effort; the arms control groups said that the ten-year research phase called for in SDI plans could reach as much as \$100,000 million, and drag on an extra three years, if cost overruns characteristic of Pentagon weapons-system development programmes occur.

SDI plans call for a decision on deployment to be made in 1983. The arms control groups' report, prepared by John Pike of the Federation of American Scientists, notes, however, that the SDI office may be unable to spend its money as quickly as it hopes to receive it. While most Department of Defense programmes manage in any given year to disburse about half of the funds appropriated in that year (and about a quarter in the following year), the SDI programme has been able actually to spend only one-third of its money in the year it was appropriated.



The costs of the US Strategic Defense Initiative and predecessor programmes, expressed in "constant" dollars at fiscal year 1986 price levels. The "deployment" costs refer to the deployment during the early 1970s of the US anti-ballistic missile system.

The arms control groups' emphasis on the budgetary consequences of Star Wars appears to be a shift in strategy. Until now, the arguments against Star Wars have centred on its technical unfeasibility and its destabilizing effects on the balance of strategic power. The budgetary argument appears designed in part to counter the Reagan administration's argument that the programme is only a research effort. "Its

massive budget will create pressures for deployment", Pike said. The report also charges that the programme will involve testing of actual anti-missile prototypes, possibly violating the ABM treaty (which forbids any testing of space-based, air-



based, sea-based or mobile land-based systems or components). According to the report, the following hardware is scheduled for testing during the research and development phase:

- **Booster surveillance and tracking system.** Components of this mid-wavelength infrared system are due to be tested on early-warning satellites in the mid-1980s; operational testing, in which the system would be used to track missiles in their boost phase, is planned for the early 1990s.

- **Space surveillance and tracking system.** Operational capability is scheduled for the early 1990s for this long-wavelength infrared sensing system, which may be able to detect thermal "signatures" of missiles in mid-course.

- **Terminal imaging radar.** A demonstration of this X-band radar, designed to discriminate between decoys and warheads, is planned for the late 1990s.

- **Lasers and neutral particle beams.** Space-based and ground tests will take place in the 1990s.

- **Pointing and tracking mechanism for nuclear-pumped X-ray lasers.** A space-based test of the pointing and tracking component is planned.

- **Ground-based rocket interceptors.** Tests are planned of the High Endo-Atmospheric Defense System (HEDS) as well as a follow-on to the Homing Overlay Experiment using smaller interceptors.

- **Rail guns.** Space-based tests of a hypervelocity launcher are planned for early 1990s.

- **Rocket-propelled space-based launchers.** Tests in space of this system, which follows the lines proposed by the High Frontier organization, are expected in early 1990s.

Stephen Budiansky

Feedstuff hormones

Europe to ban without proof?

Brussels

POLITICAL pressure from member states of the European Community on the use of synthetic hormones in farm livestock may mean that scientific opinion is overridden. While European consumer groups, led by the Bureau of European Consumers' Unions (BEUC), prepare another veal boycott over the use of growth hormones (which increase the weight of calves and beef cattle and make them more manageable), the Commission is alarmed that a scientific report on growth promoters that it has commissioned will be preempted.

In February, BEUC accused the European Commission of procrastination in producing the report on the controversial artificial growth promoters trenbolone and zeranol (which imitate the activity, respectively, of testosterone and oestradiol). The report is being drawn up by a special scientific group on anabolic agents led by Professor Eric Lamming of the University of Nottingham and was originally due last autumn. BEUC regards the delay as an attempt by the Commission to renege on earlier commitments made in the wake of the hormone scare in 1980 in Italy, when babies fed with babyfood made with veal containing synthetic hormone residues began precociously developing sexual features, not necessarily those of their own sex.

Although European farm ministers originally said they were in favour of a ban on the use of all hormones for fattening purposes, pressure from the French and US veterinary industries as well as member states where synthetic hormones are widely used, led to a subsequent Commission ban on only the most dangerous of the synthetic hormones — stilbenes and thyrostatic substances.

As well as expressing concern for consumers' health, BEUC insists that the present 700,000-tonne beef surplus in the Community constitutes an argument against the use of growth promoters in livestock on economic grounds. Only the United Kingdom, Ireland and Luxembourg use synthetic hormones. West Germany permits the use of natural hormones, but the use of all hormones for fattening purposes is banned in the remaining member states.

After looking at the evidence compiled by the Lamming group, the Community's scientific, veterinary, animal nutrition and food committees last April urged the introduction of strict precautions in using natural hormones (17 β -oestradiol, testosterone, progesterone and their less stable additives) and more effective monitoring. BEUC wants a strict system of controls as well as labelling stating clearly that the meat contains no added hormones.

The problem of the two synthetic hormones still allowed as additives, trenbolone and zeranol, is that the data are considered insufficient. Partly in answer to requests by the industry the Commission agreed to further research and reconvened the Lamming group. Its failure to report last autumn is seen by BEUC as an indication that the European Commission is bowing yet again to pressure from member states and interested parties.

European Commission officials deny this but admit that political pressure is so strong, on both sides, that scientific views may not really be taken into consideration.

The report may not now appear before the end of 1985, but, according to Professor Lamming, the reasons behind the delay are technical. Consumer concern, he says, has generated much new research into the two compounds. Analysing all the data takes time.

The evaluation of the latest data has also raised questions about the reliability and relevance of test methods. The immediate objective is to tell whether there is a threshold dose of hormones below which no effects appear, but this is more than usually complicated by doubts about the relevance of tests with laboratory species to the potential hazards of such compounds in humans.

Anna Lubinska

Danube dam stopped

WORK on the controversial Gabčíkovo-Nagymaros hydroelectric project (see *Nature* 311, 293; 1984 and 313 171; 1985) has now been halted, Mr Matyas Szuros, secretary of the Central Committee of the Hungarian Socialist Workers' Party revealed last week. This means, in effect, that the Czechoslovak government has agreed to a moratorium on the almost completed diversion and dam at Gabčíkovo. Work on the Hungarian dam at Nagymaros was stopped in 1981 for lack of funds and under pressure from Hungarian ecologists fearing irrevocable damage to the water-table of north-west Hungary, destruction of river-life and the drowning of the scenic "Danube Bend", Budapest's chief resort area.

Interviewed on Budapest radio, Szuros explained that when the agreement for the scheme was first signed in 1977, the effect on the environment could not be calculated. The two governments, Szuros said, had modified the original agreement "on the basis of mutual agreement" in 1983. This would imply that the moratorium had been agreed before the environmentalists' petition against the dam and the clamp-down on public discussion of the project at the beginning of 1984.

According to Szuros, there is now until the middle of 1985 to "analyse in a comprehensive way, carry out scientific research and assess the potential further effects on the environment" of completion of the project.

Vera Rich

Radioactive waste

Disunited states urged to act

Washington

STATES could soon face serious problems disposing of low-level radioactive waste, unless efforts now under way in the US Congress succeed in breaking the political stalemate which, for the past four years, has prevented the development of new disposal sites.

The radioactive waste materials concerned consist mostly of discarded laboratory equipment and clothing. Arrangements for the disposal of highly active wastes, such as arise in fuel reprocessing, are covered by separate legislation.

A law passed in 1980 gave states until 1 January 1986 to form themselves into regional compacts responsible for their own waste disposal, after which date the three existing disposal sites would be able to exclude waste from other regions. States lacking their own disposal sites have, however, blocked the required congressional approval allowing them to do so; in retaliation, the three states with sites have threatened to close them down completely if they are required to take out-of-region waste after next year. It is still unclear how a short-term crisis will be avoided.

Some 2.7 million cubic feet of low-level waste are produced each year in the United States, half of it by electrical utilities and half by hospitals and research institutes. The need to develop new sites will become urgent as the rate of waste production increases; roughly 40 per cent more waste will be produced each year by 2007. Most states have now reached provisional compact agreements under the low-level radioactive waste policy act, but as choosing and developing a suitable site can take five years, some urgent interim arrangements are necessary. Congress is thought unlikely to grant the necessary approval for formation of regional compacts until a solution to the immediate problem is found.

The latest attempt at a political solution is a bill introduced in the House of Representatives by Congressman Morris Udall (Democrat, Arizona). Udall's bill, which arose out of extensive consultations with state governments, would require states without disposal sites to submit within one year plans for developing sites; the states with operating sites would be obliged to continue taking limited quantities of out-of-region waste until 1993. Udall's bill, which is in the form of an amendment to the 1980 act, would also provide a standard form of agreement for compacts. But even in the bill does prove politically acceptable, in its present form it would provide only a partial solution to the growing waste problem.

There is general agreement among states that the two major sites now in operation (at Barnwell, South Carolina, and Hanford, Washington) should take less

waste in future than they do now. Under Udall's proposal they would have to take each year only 60 per cent of the amount of waste they received from outside their compacts in 1983; a third, much smaller, site in Nevada would have to take only 150,000 cubic feet of out-of-region waste. Udall makes no provision for the remaining 40 per cent, and some states are making plans for drastic action.

One widely discussed option is that power companies might be required to store their low-level wastes at power stations for five years, after which time it is hoped new sites will be nearing completion. The proposal is, not surprisingly, being resisted strongly by the utilities; the American Nuclear Energy Council, a lobbying group, is pushing for "good faith agreements" between states to ensure continued access to the existing sites. But supporters of the five-year storage idea point out that it would reduce waste production to under 60 per cent of the 1983 level and so perhaps solve the crisis.

Although not included in Udall's bill, the five-year storage plan is likely to be discussed at forthcoming meetings of state representatives where the waste issue will be tackled. There is, however, general recognition that any solution is likely to be fraught with legal complications, because agreements between states come close to the dangerous ground of balance of power between states and the federal government.

Two provisional regional compacts have already been submitted for approval in the 99th Congress and three more are expected to follow shortly. The outlines, at least, of compact agreements can be discerned for most of the country, though with negotiations at different stages. Negotiations centre on procedures for deciding where in each compact a new disposal site would be built; some compact agreements specify, for example, that all member states producing more than a certain percentage of total waste must have a site in their own territory.

The major political log-jam is in the north-east of the country, where Massachusetts and New York, both major producers, have refused to form compacts with New England states. Massachusetts, under a state law known as chapter 503, cannot join a compact or approve a site decision until a public referendum is held — which cannot be until mid-1986, after the legal deadline for formation of compacts. Both New York and Massachusetts may be forced to consider temporary storage facilities and to impose draconian waste-reduction measures that would go beyond the voluntary efforts made so far. As for what will happen in the longer term, the answer is, in the words of one analyst following the issue, "anybody's guess".

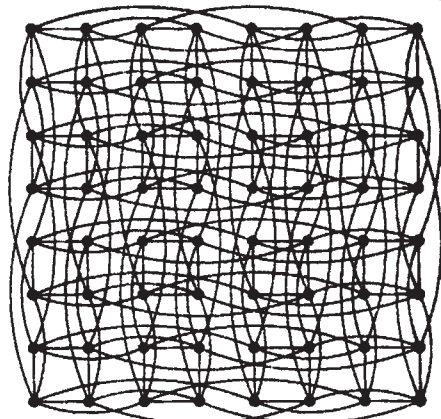
Tim Beardsley

Supercomputers

Intel's hypercube architecture

Washington

INTEL, the computer-chip manufacturer, is coming out with the first of what it hopes will be a new generation of supercomputers, inexpensive machines that offer high computational power by hooking many small computers together in parallel operation. The Intel machines, which will initially be offered in configurations consisting of 32, 64 or 128 separate computers, or "nodes", are to be aimed specifically at scientific users, particularly those tackling large-scale



A hypercube connects $N = 2^n$ small computers, called nodes, through point-to-point communication channels in the Cosmic Cube. Shown here is a two-dimensional projection of a six-dimensional hypercube, or binary 6-cube, which corresponds to a 64-node machine.

modelling problems in areas such as fluid dynamics and circuit simulation.

The chief attraction of the Intel computers is certain to be their price, which will range from \$150,000 for the 32-node model to \$520,000 for the 128-node. The largest has a peak capacity of 10 million floating-point operations per second (MFLOPS), or about 25 times that of Digital Equipment Corporation's VAX 11/780, which costs about \$225,000. Perhaps the most familiar supercomputer, the Cray-1, costs \$5-10 million. Intel has announced that its computers will be available this summer.

Unlike the other parallel-processing supercomputers on the market, the Intel machines break totally with the conventional computer architecture based on executing a series of instructions in sequence. Each node consists of a separate central processor, floating-point processor and 512-kbyte random-access memory. Memory is not shared between the separate nodes. Each node can communicate with those adjacent to it in a hypercube layout (see diagram). In essence, the idea is to increase performance by stringing together many cheap processors, taking advantage of the recent advances in Very Large Scale Integration technology that have sent prices plummeting, rather than spending more and more on ultimately diminishing returns in attempting to improve performance of individual processors.

Intel's problem with this, its first entry into the marketing of complete computer systems, is that people do not know how to program highly-parallel processors. Although the company says it is encouraging third parties to develop applications software, it is offering only an operating system initially. Conventional supercomputers, such as the Cray, allow users to write their programs in the ordinary sequential manner; smart Fortran compilers (for example) are then employed to pick out the steps that can be executed simultaneously. The Cray-type parallel processor operates most efficiently when tackling, for example, vector arithmetic operations; its parallel processors can simultaneously perform the requested operation on each element of the vector. The inherent disadvantage of this approach, however, is that the overall execution is still sequential, and the high speed gained in vector operations inevitably runs into a "bottleneck" when single scalar operations are reached during the program.

To program the Intel-type machine successfully, the problem has to be split into independent pieces that each node can tackle simultaneously. In fluid dynamics problems, for example, each node can be assigned one sector of coordinate space; since the underlying physical laws involve only nearest-neighbour interactions of particles, each sector can be computed independently; the only communication between nodes that is necessary is the result at the edge of each node's sector.

In dealing with such problems, each node is used to its fullest, unlike the Cray-type machine which operates in practice only at 5-20 per cent of its peak capacity. (The Cray-1 has a peak capacity of about 160 MFLOPS.)

In justifying its somewhat daring move, Intel points to a potential market for such "near supercomputers" of \$100 million now, reaching ten times that in 1990. Supercomputers now account for \$300 million of the scientific computing market, estimated to be at least \$2,000 million. Intel clearly also has its eyes on the National Science Foundation's plans to support the establishment of supercomputer centres and to purchase time on existing machines in response to a considerable perceived demand for scientific supercomputing.

The Intel machines are based on the design developed by Charles Seitz, a researcher at California Institute of Technology. Intel is licensing the design from Caltech.

Stephen Budiansky

Correction

THE article "Corporate plans all the rage" (*Nature News* 31 January, p.341) incorrectly states that the Ordnance Survey is the responsibility of the Ministry of Defence rather than the Department of the Environment. □

Japan Prize

First awards go overseas

Tokyo

JAPAN'S answer to the Nobel prize was launched on 15 February with the announcement of the first two Japan Prize laureates. Honoured by the Science and Technology Foundation of Japan were Ephraim Katchalski-Katzir of the Weizmann Institute of Science, Israel, and John Robinson Pierce, professor emeritus of Stanford University in the United States.

The Science and Technology Foundation is itself the creation of its president, Konosuke Matsushita, founder of the vast Matsushita Electrical Industries, Japan's fifth biggest company. Matsushita is well known as a philanthropist and even philosopher; he established the spiritual and educational PHP (peace and happiness through prosperity) movement that has gained enormous popularity in Japan. Although Matsushita donated 3,000 million yen (\$12 million) to establish the Prize, he has stayed very much behind the scenes since — the foundation is officially a non-profit foundation endorsed by the Cabinet for the awarding of medals of honour. The foundation's funds have, however, made it possible to give a prize of ¥50 million (\$200,000) with the medal. The Japan Prize thus rivals the Nobel prize financially; the intention is that with the passage of time it will also rival it in prestige.

The Japan Prize's aim is a little different from that of the Nobel prizes: it is intended for those who have achieved breakthroughs that can clearly be put to work in new ways that will "contribute to world peace and prosperity", rather than at discoveries in basic science. Perhaps the prize will help in some way to overcome Japan's inferiority complex that the development of original new technology is in some sense a less creative endeavour than research in pure science.

For this first year, three target areas — biotechnology, medical engineering, and information and communication — were selected and nominations of scientists in these fields sought from well-known academics around the world. Some 432 recommendations for 291 candidates were received, roughly two-thirds of them from outside Japan. A 24-member committee, split into sub-groups for the three areas, then chose just two laureates.

Considerable surprise was expressed (by members of the Japanese press, at least) that no Japanese was chosen for the prize even though Japan has made a considerable contribution in the three areas. The simplest explanation, other than plain modesty, seems to be that the principal Japanese workers in these areas were all members of the selection committee.

In the biotechnology category, Ephraim Katchalski-Katzir was awarded the prize

for the development of the immobilized enzyme systems now in widespread use in bioreactors and bioanalysers, which have done much to trigger the present biotechnology boom. He showed in the 1960s (see for example *Nature* **188**, 856; 1960) how the charge distribution and the hydrophobic and hydrophilic properties of enzymes affect their interaction with a carrier agent and their catalytic activity, thus making it possible to define criteria for selection of proper enzyme carrier materials. Japanese companies now lead the world in immobilized enzyme technology and the prize may thus partly acknowledge a debt to his pioneering work.

Professor Katchalski-Katzir is now 69 and a professor at the Weizmann Institute. He also served as Israel's president from 1973 to 1978, continuing a tradition which began with Chaim Weizmann himself that

prominent scholars should hold the presidency.

In the information and communication category, John R. Pierce received the prize for a number of studies, among them his leading role in the development of pulse code modulation theory which has made possible the digital communications technology now coming into use everywhere. His other key discoveries include the Pierce gun, the key to the design of practical travelling wave tubes and now in use in microwave communications systems, and the Pierce loop, which has made possible the rational design of Local Area Networks. Professor Pierce, now 75, has been guest professor at the Stanford University's Center for Computer Research on Music and Acoustics since his retirement as head of research at Bell Laboratories.

Alun Anderson

Chinese reactors

China plans for independence

CHINA'S nuclear industry must switch from military to civilian aims, Li Peng, vice premier of the State Council, urged last month. Addressing a "work conference" of the Ministry of the Nuclear Industry, he called for the ministry to direct its main efforts to nuclear power generation, while at the same time to diversify by providing services on a contract basis to other branches of industry. This could entail a major change from the defence-orientated stance in which good economic returns were desirable but not the prime necessity to a more cost-effective approach.

Li Peng's remarks have been widely publicized in the Chinese media, and contrast oddly with the last major coverage of nuclear matters — the twentieth anniversary, last October, of the first Chinese nuclear bomb. (Among more overtly political issues, it was noted, on that occasion, that the Lop Nor test site has made a good ecological recovery, with burgeoning grass and gazelles.) Much of his speech, however, related rather to the reform of the Chinese economy; the need to develop "horizontally" and the role of technology transfer in achieving the "four modernizations". As an example, he urged that the technology for exploiting uranium mines developed by the Ministry of the Nuclear Industry could be transferred to the coal and non-ferrous ore sectors. He also took the opportunity of announcing the government's decision on the vexed question of ministerial responsibility in nuclear power development. Responsibility for the construction of large-scale nuclear power stations, Li Peng said, would rest with the Ministry of Water Resources and Electric Power, while the construction of the "nuclear island" would be borne by the Ministry of the Nuclear Industry.

Nuclear power in China, Li Peng suggested, must gradually become a completely Chinese matter. The Ministry of the

Nuclear Industry already possessed, he said, a complete system for processing nuclear materials "from the exploration and exploitation of uranium mines to the post-processing of irradiated fuel". This expertise must be used so that nuclear fuel can be supplied to the power stations by Chinese enterprises, and so that a complete nuclear fuel cycle can be established.

The publication of Li Peng's speech was delayed for three weeks to coincide, on 24 January, with the start of construction of what was officially described as the first nuclear power plant designed and built exclusively by China. This is the 300 MW pressurized-water station at Qinshan, which will serve the east China industrial network centered on Shanghai. The Qinshan station is expected to provide a "complete range of experience for building power plants", and hence will have a key role in Chinese plans to construct a total nuclear capacity of 10 GW by the end of the century.

Following Li Peng's lead, Chinese spokesmen have stressed the indigenous nature of the project, and rumours that China planned to purchase two nuclear-powered generating facilities from the Soviet Union under the 1986-1990 Sino-Soviet economic and trade agreement have been strenuously denied by the Chinese Foreign Trade Ministry.

But the Qinshan project may be less than exclusively Chinese. The reactor pressure vessel and reactor main coolant pump will be built by West German and Japanese companies to Chinese designs, and the design of all major equipment has been checked by consultants from the United States and Italy. Moreover, according to Zhou Ping, vice minister of the Nuclear Industry, the experience gained at Qinshan will enhance China's capacity to absorb imported nuclear power technology, paving the way for the import of large complete generating sets.

Vera Rich

French reactors

Problems only of plenty

FRENCH nuclear power strides on — even though the major nuclear constructor, Framatome, is tottering and the first commercial-scale fast breeder, Superphénix, is suffering problems that could delay its start-up by one or two years.

First, the good news. Last year, French 900-MW pressurized water reactors (PWRs), the bulk of the stations now operating, achieved an availability (energy delivered over energy theoretically available) of 79 per cent. These would seem to be extraordinary figures in relation to the results of only a couple of years ago, when availability was in the sixties, and again confirm the claims of Electricité de France (EDF), the national utility, that these were only "teething problems" in reactors which were mostly very new. The first two 1,300-MW PWRs, Paluel 1 and Paluel 2, also came on line last year and reached full power in late December bringing — with the other reactors — some 178 TWh of nuclear energy into the French grid in 1984 (compared with British nuclear power production in the same year of 34 TWh).

The trouble now is that with many more PWRs under construction, France really needs no more electricity, and Framatome is in dire need of diversification. The problem can be solved only by finding new industrial partners (or products), by selling reactors abroad or by exporting electricity.

All options are open. On electricity exports, EDF nearly doubled to 25 TWh the amount of electrical energy exported from France in 1984, and plans to do better in 1985 with the establishment of a high-power cross-Channel link with the British grid. On the export of reactors, Framatome is bidding hard for contracts in Egypt and in Israel. France, it is believed, is prepared to let Israel buy its reactors without demanding that Israel sign the non-proliferation treaty — the stumbling block for a deal with the US company Westinghouse in the 1970s.

Meanwhile, Superphénix, the great white hope for European nuclear power in the next century, has been troubled by vibrations in certain components in the sodium cooling circuit, now under test before what was to be the fuelling of the reactor this summer. The elements are baffles in the stainless-steel "cauldron" in which the fuel elements sit, and they appear to vibrate, when the sodium is introduced and pumped. The problem is whether to rebuild the baffles, which could cause a lengthy delay in the operation of the reactor, or to load the fuel and hope the problem goes away when the reactor reaches its working temperature of 425°C, some 75°C above the temperature at which preliminary tests have been conducted.

Robert Walgate

Embryo research

British Commons vote for ban

IN Britain last week, the House of Commons passed by a large majority (238 votes to 66) Mr Enoch Powell's emotively titled "Unborn Children (Protection) Bill" (see *Nature* 7 February, p.417), challenging both government and opposition alike. If the bill becomes law, all experiments on human embryos will be banned and it will be illegal to have in one's possession an embryo produced by *in vitro* fertilization (IVF) unless it is to be implanted into the uterus of a named woman with the aim of bringing the embryo to full term. For this, the permission of the Department of Health will be necessary.

Mr Kenneth Clarke, Minister for Health, represented the government's view, which is to introduce comprehensive legislation at some future date, based on the findings of the Warnock Committee (see *Nature* 312, 389; 1984). Despite pressure from backbenchers in his own party, Mr Clarke refused to set a date for such a bill. The Warnock Committee took nearly two years to produce its report and the government has now invited comments on it from interested organizations. More than 120 such depositions have so far been received among which there is "little unanimity". Mr Clarke said that all these views must be considered before the drafting of any legislation, which will encompass much broader issues than the current bill, including surrogacy and the intricate problems of the legitimacy of children born through IVF or other techniques.

Mr Michael Meacher, the Labour opposition spokesman on health, somewhat unusually on the same side as the government, also spoke against the bill, arguing in favour of regulated research and pointing out the huge procedural task involved for the Department of Health in assessing IVF applications. He also warned of the wider implications of the bill for contraception and abortion.

Those in favour of the bill, however, had simpler points to make. Mr Powell's view is that embryos are people, and that killing people is wrong; he acknowledged last week that his bill would impede research, at least until other means are found, but said that this is the lesser evil. The spectre of the Nazi scientist was raised by several Members of Parliament (MPs), who felt that a blanket prevention of embryo research is safer than regulated licensed experiment. Mr Ian Paisley and Mr Norman St John Stevas both supported the bill on religious grounds, arguing that it is morally wrong to create life in order to discard it later.

Arguments against the bill plainly failed to impress most MPs. Ms Clare Short pointed out that the "test-tube" babies alive in Britain today would not have been born had the bill been law before the techniques had been developed. Several MPs, including Mrs Renee Short, Mr William

Hamilton and Mr David Couch (the parliamentary lay member of the Medical Research Council), described the potential benefits of IVF research in elucidating the causes of genetic defects and investigating male and female infertility, as well as the need to improve the success rate of IVF techniques. Despite these arguments, 170 Conservative MPs voted in favour of the bill, including the Leader of the House of Commons and the Chief Whip, on a free vote.

Next, the bill must pass through the committee stage. The government's failure to prevent the bill so far and the bill's popularity with its own MPs will make it more difficult for it to delay or prevent the bill from passing through this notoriously thorny procedural step. The bill will also have to be passed by the House of Lords

before it can become law. The government could remove much of the bill's support by announcing a definite timetable for its own legislation based on the findings of the Warnock report. Many of the Conservative MPs who voted for the bill last week would prefer government-sponsored legislation, but, in the absence of a definite date, would prefer to ban experimentation altogether than allow it to continue under present legislation.

Lady Warnock, speaking this week at King's College London, said that if the bill became law, the National Health Service IVF programme in Britain would end. She also explained that the minority opinion dissenting from her report's recommendation of a 14-day limit for embryo research and the reason why "people genuinely believe that science is up to no good" stemmed from the fear that the limit would not be adhered to rather than from opposition to experiments as such.

Maxine Clarke

French influence on Britain

PROFESSOR Jérôme Lejeune, the French geneticist who discovered the trisomy of chromosome X21 that causes Down's syndrome, seems to have had a great influence over British Members of Parliament (MPs) in their vote last week on human embryo research (see above).

A week before the debate, Lejeune addressed MPs and argued strongly that the research was unnecessary. So, repeatedly in the debate, MPs were quoting this "most eminent geneticist" in justification of their votes. Lejeune, brought to Britain for the debate by the anti-abortion lobby, is described by some British scientists as a "very religious and very conservative" Roman Catholic.

In France, Lejeune is an active member of the pro-life lobby *Laissez-les Vivre*. Last week, he was quoted by MPs such as Mr Norman St John Stevas (who backed the bill), as saying that "[embryonic research] is not necessary" and by Sir Bernard Braine, who said that "It is clear from what he says that it is misleading nonsense to assert that experimentation up to 14 days can add anything at all to our knowledge of diseases such as muscular dystrophy... or cystic fibrosis", as by then the relevant tissues have not developed.

According to Sir Gerald Vaughan MP, Lejeune claimed at the pre-debate meeting that benefit from embryonic research was "very unlikely", and that "when one considers congenital conditions, the most profitable lines of research all lie in the chemical and vitamin field, many of them dealing with the mothers and not with the embryo".

This is Lejeune's line of research at the University of Paris, where he heads a group (at Paris V) investigating the role of "monocarbons", or C_1 -radicals active in the developing brain, in causing Down's

syndrome.

Strangely enough, however, Lejeune's impact in France has been less than it appeared in Britain last week. The director of the French medical research council's research unit on reproductive physiology and psychology, Dr Emile Papiernik describes Lejeune as "a descriptive scientist, not in the field of experimentation". He added that in Britain "there seems to be a very conservative feeling at the present time".

Lejeune, speaking from Paris on Monday, said he was "very honoured" but surprised to have had an influence over British MPs. He explained that his opposition to experimentation is on two levels: first, he believes that the fertilized egg is a human being, and that people should not be experimented upon; second, he "had not seen any argument" that convinced him that embryonic research would be beneficial.

Arguments in the Warnock report that the research could help cure Down's syndrome, haemophilia and muscular dystrophy were "just scientifically wrong", and anyway, he asks, why not do the research on embryonic material from animals like dogs and pigs?

Moreover, in Lejeune's view, *in vitro* fertilization (IVF) is not a treatment for infertility (whereas a repair of damaged fallopian tubes would be), but a cure for "childlessness": "There is a semantic distortion" in the debate, he says. Embryo research is not needed to treat infertility.

Lejeune has no objection to IVF, although he does object to the cryogenic preservation of embryos ("just a trick to have some material to hand").

He believes that many genetic diseases will be treatable with drugs without aborting a genetically disordered embryo.

Robert Walgate

SCOPE and nuclear war

SIR — I suppose it would seem odd to some people that “the nuclear winter subcommittee of the International Council of Scientific Union’s Scientific Committee on Problems of the Environment (SCOPE), which is due to publish its own study next June, should have been able to afford time for a five-day workshop at Bellagio to draft and put out a statement which, on the face of things, will compromise the impartiality of its own work” (*Nature* 20/27 December, p.696). But not to the people who know that the subcommittee did *not* afford time, did *not* draft and did *not* put out the statement.

The official statement indicates explicitly that it was an “*ad hoc* group of scientists and religious leaders . . . gathered at the invitation of ICSU and the Inter-Faith Academy of Peace”. In addition, it should be noted that the group did *not* include Abdus Salam.

The subcommittee, which is concerned with the environmental effects of nuclear war, not specifically “nuclear winter”, is continuing with its studies and meetings. The impartial report of its study will be drafted in June for submission to the SCOPE General Assembly in September.

F.W.G. BAKER
(Executive Secretary)

International Council
of Scientific Unions,
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75016 Paris, France

Science spending

SIR — John Maddox is right when he claims (*Nature* 31 January, p.347) that modern “big” physics and astronomy are inevitably expensive, but his idea that we should persuade research communities and governments worldwide deliberately to slow the rate of progress in these fields is remarkably parochial. Many of these other governments, unlike ours, support basic research in an enthusiastic way which suggests that they really do believe their own public statements about its importance. Most of them also spend much smaller fractions of their research and development budgets on secret defence research than us. As a result, our highly productive research scientists work at a relative disadvantage, and the government now demands that these scientists must select those of their own colleagues whose work should be stopped.

A natural reaction to this “divide and rule” proposal is the sad spectacle of a television debate in which an eminent biology professor decries the value of high-energy physics and asks for its funds to be redirected to biology. This type of proposal is appropriate only if we accept that we really cannot afford properly to support

our current basic research programmes. Surely this is incorrect, and we must persuade the government that it is essential both to support CERN and to fund all of the high quality medical research that is now under threat?

John Maddox’s proposal to slow or abandon some work may have some merit, but not in relation to “big” physics or astronomy. Defence spending is much bigger and more futile, and we have now achieved international armouries adequate to kill us all many times. Given a choice between new information on the relationships between elementary particles and an “improved” armoury capable of killing us all with even greater certainty, I know which I would ask my government to choose.

BOB MICHELL

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Star wars

SIR — I wish to correct two errors in your leading article “How to talk about star wars” (*Nature* 3 January, p.1).

You refer to a study by my “committee operating under the flag of the Union of Concerned Scientists in the United States”. In fact, the study that Drs Philip Farley, David Holloway and I performed at Stanford was an independent one and was published by the Stanford University Center for International Security and Arms Control with the title *The Reagan Strategic Defense Initiative: A Technical, Political, and Arms Control Assessment*.

More importantly, in contrast with the statement in your leading article, the size of the constellation and the number of space-based laser battle stations had little to do with the main thrust of our arguments. Although that particular issue has triggered much discussion and controversy in the public press, I believe it to be of only minor significance in analysing the potential and the problems of the Strategic Defense Initiative. As our Stanford analysis emphasized, the issues of primary concern have to do with the strategic political and arms control impact of the star wars programme as originally proposed and with operational problems faced by a fully integrated system operating against an offence that can respond with diverse and effective countermeasures to a defensive deployment. The general conclusion we stated in our report was:

“Our analysis raises grave doubts, on technical and strategic grounds, that substantial acceleration or expansion of ABM [antiballistic missile] research and development is warranted or prudent. Deliberation and restraint are imperative not simply because of the enormous costs of the proposed near-term SDI [Strategic Defense Initiative] research and technology program, but because the strict limitation of ABM deployments is one of the few

points of real agreement reached in the US-Soviet dialogue about nuclear war and arms control. It has practical consequences of great importance for the effectiveness of our deterrent, for such fragile strategic stability as has been achieved, and for our prospects of avoiding nuclear war.

“If defensive systems are to contribute to a safer and more stable strategic relationship between the United States and the Soviet Union, they will have to be embedded in a strict arms control regime that limits offensive systems. Technology alone will not solve the political problem of managing the strategic relationship with the Soviet Union.” SIDNEY D. DRELL
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Stanford, California 94305, USA

Statistics and philosophy

SIR — George Gale¹ looks for the theory of science to develop from an academic philosophy of science but fails to see statistics bordering philosophy, science and mathematics. Hoping to clarify the nature of probability and its role in logic and science, philosophers have studied theories of probability that are vital to different schools of statistics. That statistics goes beyond probability shows up in the areas statisticians study — from the design of experiments² to the weighing of evidence³ to informal data analysis⁴ to what makes a good visual display⁵.

First-rate statistical innovations often result when good statisticians collaborate with scientists⁶. While designing experiments, weighing evidence, analysing data informally and summarizing data with useful displays do not form a theory of science, these individual accomplishments show that statisticians have much to say about the theory and practice of science. Perhaps embattled philosophers of science with an empirical bent can find friends among statisticians. Good philosophers, collaborating with statisticians, might even produce first-rate philosophy.

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1. Gale, G. *Nature* 312, 491 (1984).
2. Fisher, R.A. *The Design of Experiments* (Oliver and Boyd, Edinburgh, 1935).
3. Good, I.J. *Probability and the Weighting of Evidence* (Charles Griffin & Co., London, 1950).
4. Tukey, J.W. *Exploratory Data Analysis* (Addison-Wesley, Reading, Massachusetts, 1977).
5. Cleveland, W.S. *The American Statistician*, 38, 261 and 270 (1984).
6. Box, G.E.P. *Technometrics*, 26, 1 (1984).

Totals wanted

SIR — Would those who publish a sequence please include the totals of amino acids (or bases). J.A.D. EWART

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Nitrates, nitrites and gastric cancer in Great Britain

from David Forman, Samim Al-Dabbagh and Richard Doll

Nitrate and nitrite were measured in the saliva of two populations who differed in their risk of developing gastric cancer. Surprisingly, the levels of both ions were significantly higher in the low-risk group.

THE past few decades have seen a progressive increase in the general level of nitrate in the environment. This has been due partly to the formation of nitrogen oxides by fuel combustion and their deposition as a component of acid rain¹, partly to increases in sewage recycling, but mainly to the increased use of nitrate based fertilizers. The result has been an increase in nitrate accumulation in some root and leafy vegetables and a leaching through of nitrate into freshwater supplies² and hence into drinking water³⁻⁶. Vegetables and drinking water provide two of the main sources of human exposure to nitrate and nitrite, the other major source being the preservatives added to meat to prevent botulism and to enhance colour. The permitted levels added to meat have been progressively decreased in recent years⁷, but overall there has been a significant net increase in exposure due to the increased amounts in vegetables and water⁸.

Conversion

Concern about nitrate is not related to the ion itself, but to the fact that certain species of bacteria found in the mouth, and sometimes in the stomach and bladder, can enzymatically reduce nitrate to nitrite⁹. Such nitrate-derived nitrite is the major source of exogenous nitrites for most humans. Apart from the specific problem of nitrite-induced methaemoglobinaemia, which is hardly ever seen in the United Kingdom¹⁰, nitrite represents a potential hazard because of its participation in nitrosation reactions giving rise, with appropriate substrates, to *N*-nitroso compounds most of which are strongly carcinogenic in animals¹¹. It has therefore been suggested that an increase in nitrate exposure may represent an increased cancer risk through the production of *N*-nitroso compounds^{12,13}.

The site most commonly regarded as being at risk from endogenous *N*-nitroso compound synthesis is the stomach. Stomach acidity favours the nitrosation reaction¹⁴ and many *N*-nitrosamides are

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IMAGE
UNAVAILABLE
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REASONS

In the United States the absence of nitrates and nitrites is a selling point. Most processed meat products, especially pork, contain nitrate or nitrite additives.

known to be locally acting in animal experiments^{13,15}. There is also a small amount of direct human evidence to implicate nitrate exposure directly. International correlations relate high levels of nitrate intake to gastric cancer mortality¹⁶ and nitrate exposure has been associated with gastric cancer in areas of England¹⁷, Colombia¹⁸, Chile¹⁹, Japan²⁰, Denmark²¹, Hungary²² and Italy²³. None of this evidence is, however, more than suggestive²⁴ and some has been contradicted by other studies^{25,26}. Thus, whereas some writers now view nitrate as a likely human risk factor for gastric cancer^{13,16}, others believe there is no compelling human evidence to show that it is^{12,24}.

As a result of these shortcomings in the human data and the concern that has been expressed about nitrate exposure, we designed a study to look at the relevance of nitrates to gastric cancer in the United Kingdom. We took advantage of the long-standing, regional differences that are

known to exist within Britain for gastric cancer mortality²⁷. Our aim was to compare populations from consistently high mortality areas with those from consistently low mortality areas to see if they differed in their exposure to nitrate. To estimate the exposure, we measured nitrate and nitrite levels in samples of saliva. Some 25 per cent of exogenous nitrate is actively taken up by the salivary ducts from the circulation and about 20 per cent of this is reduced by oral bacteria to nitrite^{28,29}. This nitrite, formed from reduced nitrate, in fact represents approximately 80 per cent of total exposure to nitrite¹², the remainder coming directly from exogenous sources. Salivary measures should, therefore, be a good indicator of nitrate and, particularly, nitrite that will be conveyed to the stomach.

Populations were drawn from four regions of Britain — Wales and the North-East (high gastric cancer mortality regions) and Oxford and the South-East (low

Table 1 Characteristics of study populations

		Low cancer risk			High cancer risk		
		Area I	Area II	Areas I & II	Area III	Area IV	Areas III & IV
Area SMR for gastric cancer*	M	79	59 & 76		138 & 162	156	
	F	66	80 & 67		160 & 147	130	
Regional SMR for gastric cancer†	M	80	91		112	121	
	F	88	83		114	132	
Numbers in study‡							
Full sample		220	202	422	209	205	414
Refined sample		120	84	204	90	86	176
Fasting sample		14	15	29	14	24	38
Refusals (%)		4	6	—	4	5	—
Males (%)§		38 (44)	41 (36)	39 (41)	36 (28)	37 (30)	36 (29)
Females (%)		62 (56)	59 (64)	61 (59)	64 (72)	63 (70)	64 (71)
Age§ 15-34 years (%)		38 (39)	35 (32)	36 (36)	34 (32)	45 (41)	40 (36)
35-54 years (%)		45 (44)	44 (38)	45 (42)	43 (42)	43 (48)	43 (45)
55-74 years (%)		17 (17)	21 (30)	19 (22)	23 (26)	12 (12)	17 (10)

Gastric cancer risk is defined here as high or low when the age standardized mortality rate (SMR) for the individual town or city is high (130 or above) or low (80 or below) for both sexes and when the appropriate regional SMR shows a trend in the same direction. Collecting areas were outpatient clinics at hospitals in the towns and cities as follows: I, Oxford (Oxfordshire RHA); II, Canterbury & Eastbourne (SE Thames RHA); III, Sunderland & Hartlepool (Northern RHA); IV, Bangor & Llandudno (Wales).

* Area SMRs for 1969-73 obtained from Office of Population Censuses & Surveys microfiche³⁰ for the following county boroughs: Oxford, Canterbury, Eastbourne, Sunderland and Hartlepool; and for Caernarvon Urban Aggregate (which covers Bangor and Llandudno).

† Regional SMRs for 1981 from Office of Population Censuses & Surveys publication⁴⁵ for Wales and the Oxford, Northern, and South-East Thames Regional Health Authorities. Using the RHA figures was more appropriate than using those for standard regions as the latter cover a larger area around the city or town of interest.

‡ Numbers given for full sample are those participating after exclusion of those refusing and those ineligible due to age or area of residence. Those in refined sample are after exclusion of those having eaten or drunk in the 2 h prior to sample. Those in fasting sample are after exclusion of those having eaten anything on day of sample or drunk in the 2 h prior to sample.

§ Numbers in parentheses are percentages in refined sample.

Table 2 Mean nitrate and nitrite concentrations (nmol per ml) in saliva of the four study populations

		Low risk		High risk		All low	All high
		Area I	Area II	Area III	Area IV	Areas I & II	Areas III & IV
Nitrate:							
All samples		208.3 (180.5-240.4)	157.3 (142.4-173.7)	107.2 (93.7-122.6)	107.9 (94.8-122.8)	— —	— —
		$P < 0.01^*$		NS†			
Refined samples		171.5 (140.8-209.0)	149.5 (128.0-174.8)	97.0 (77.5-121.4)	116.8 (95.3-143.2)	162.1 (142.0-185.1)	106.3 (91.3-123.5)
		NS*		NS†		$P < 0.0001^‡$	
Fasting samples		— —	— —	— —	— —	169.0 (120.0-237.9)	92.5 (67.8-126.3)
						$P < 0.05^‡$	
Nitrite:							
All samples		123.7 (112.0-136.6)	94.6 (85.0-105.3)	69.1 (62.4-76.4)	51.4 (45.8-57.7)	— —	— —
		$P < 0.001^*$		$P < 0.001^†$			
Refined samples		111.1 (96.1-128.3)	86.3 (73.6-101.3)	76.1 (64.6-89.7)	58.7 (47.8-72.0)	100.2 (89.8-111.7)	67.0 (58.8-76.4)
		$P < 0.05^*$		NS†		$P < 0.0001^‡$	
Fasting samples		— —	— —	— —	— —	80.8 (61.4-106.4)	44.7 (34.1-58.4)
						$P < 0.005^‡$	

Definitions of the four study areas and of high and low risk populations as in Table 1. Values are geometric means (95% confidence interval). Refined samples are those from subjects who had nothing to eat or drink in the 2 h prior to sampling, and fasting samples are those from subjects who had nothing to eat on the day and nothing to drink in the 2 h prior to sampling.

* P -values from two-sided t -tests comparing area I with area II.

† P -values from two-sided t -tests comparing area III with area IV.

‡ P -values from two-sided t -tests comparing low risk population with high risk population.

gastric cancer mortality regions). Localities were chosen within these regions on the basis of having a high (or low) standard mortality rate (SMR) for gastric cancer for both males and females over the period 1969-73. Published mortality figures for this quinquennium by local

authority area are available from the Office of Population Censuses and Surveys³⁰. Between 200 and 250 residents from each of these regions who were visiting hospital out-patients departments (that is, friends or relatives of people attending the hospital as patients and not patients themselves)

were invited to participate in the study. As far as possible, all visitors in a waiting room for a particular clinic were asked to participate, apart from people aged under 15 years or over 75 years and people not resident in the area. The refusal rate was about 5 per cent and did not vary

Table 3 Salivary nitrate and nitrite concentrations (nmol per ml) by sex (a) and age group (b)

a, Sex:	Low risk	High risk	P* (L vs H)
Nitrate			
Males	158.6 (129.2-194.6)	84.8 (62.2-115.6)	<0.001
Females	164.6 (138.2-196.1)	116.5 (98.1-138.2)	<0.01
P† (M vs F)	NS	NS	
Nitrite			
Males	106.4 (90.9-124.5)	59.2 (48.5-72.4)	<0.0001
Females	96.1 (82.8-111.5)	70.5 (59.7-83.2)	<0.01
P† (M vs F)	NS	NS	
b, Age:	Low risk	High risk	P* (L vs H)
Nitrate			
15-34 yr	126.4 (101.8-157.1)	78.4 (61.3-100.2)	<0.005
35-54 yr	167.3 (136.0-205.9)	135.0 (111.0-164.2)	NS
55-74 yr	229.8 (177.4-297.7)	108.0 (70.0-166.5)	<0.005
P‡ (age trend)	<0.001	<0.05	
Nitrite			
15-34 yr	77.9 (64.6-93.8)	45.8 (38.3-54.8)	<0.0005
35-54 yr	100.7 (86.4-117.3)	81.8 (67.4-99.3)	NS
55-74 yr	150.0 (120.4-186.7)	86.9 (62.1-121.8)	<0.01
P‡ (age trend)	<0.001	<0.001	

Values are geometric means (95% confidence interval). NS, not significant.

* P value from two-sided *t*-test comparing low risk with high risk populations within each sex and age group.

† P value from two-sided *t*-test comparing males with females within each population.

‡ P value for significance of linear regression for age group on log nitrate or nitrite concentration.

Table 4 Salivary nitrate/nitrite concentrations (nmol per ml) by social class (SC)

	Low risk	High risk	P* (L vs H)
Nitrate			
SC I & II	218.3 (169.8-280.6)	103.8 (77.1-139.6)	<0.0005
SC III	138.7 (111.7-172.1)	126.6 (100.3-159.7)	NS
SC IV & V	130.9 (101.7-168.4)	73.8 (52.1-103.6)	<0.01
P† (trend)	<0.01	NS	
Nitrite			
SC I & II	112.9 (92.2-138.1)	62.8 (49.3-80.0)	<0.0005
SC III	94.1 (79.5-111.4)	77.2 (62.2-95.6)	NS
SC IV & V	82.0 (64.6-104.2)	63.7 (46.5-87.4)	NS
P† (trend)	=0.05	NS	

Values are geometric means (95% confidence interval). Social class groupings as defined by the Registrar General's classification of occupations, 1981⁴⁶. In these populations the percentage in each social class grouping in low and high risk areas respectively were: SC I & II, 27% and 22%; SC III, 51% and 53%; SC IV & V, 22% and 25%.

* P value from two-sided *t*-test comparing low risk with high risk populations within each social class group.

† P value for significance of linear regression for social class group on log nitrate or nitrite concentration.

significantly between the regions.

All participants provided a sample of about 1 ml of saliva and completed a short questionnaire which inquired about a number of socio-economic features and dietary habits. The general characteristics of the four populations are shown in Table 1. For some of the analyses, the populations were refined by excluding anyone who had eaten or drunk within two hours before giving saliva. This is because consumption of food or drink generally leads to a rapid fluctuation in salivary nitrate and nitrite levels, which reach a peak and then fall again within about two hours of consumption³¹. The two hour or longer fast, although not producing a true baseline, does allow for standardized comparison of different populations. As can be

seen from Table 1, the four populations, both full and refined, are broadly similar to each other in terms of sex and age distribution, with a slightly increased proportion of females in the refined population for the high risk area. For some analyses, it was possible further to exclude from the refined population those who had anything at all to eat on the day of sampling (and who did not drink in the preceding two hours). The numbers in this fasting population are also shown in Table 1.

Saliva was collected into glass tubes containing 1 M sodium hydroxide as a preservative. The tubes were then kept in the dark at room-temperature until being returned to Oxford for analysis, which was always within one month of collection. Under these conditions, there is no

degradation of the sample³². Analysis was carried out using bacterial reduction of nitrate to nitrite and a modified Griess procedure for nitrite determination as described previously³².

Significant difference

The mean salivary nitrate and nitrite concentrations for the four regions are shown in Table 2. When comparing all the samples, there are some significant differences, when looking at one high risk area with the other, or one low risk area with the other. However these largely disappear (except for a borderline significance for nitrite between the two low risk areas) when considering the refined samples, consisting of those subjects who had nothing to eat or drink in the two hours before sampling. It thus seemed reasonable to pool the refined samples for both the high risk and both the low risk areas (Table 2). The direct contrast between the high risk and low risk populations shows that the low risk population had significantly increased salivary nitrate ($P<0.0001$) and nitrite ($P<0.0001$) levels. The means in the fasting populations are also shown in Table 2. These results still show a significant difference in the levels, with high values in the low risk population (nitrate $P<0.05$; nitrite $P<0.005$).

Using refined samples, no major differences are observed when comparing individuals from whom samples had been taken at different times of day or at different seasons of the year (D.F. and S.A.I.-D, unpublished observations).

Table 3 shows salivary concentrations by sex and age. Sex makes no obvious difference, but increasing age is generally associated with an increase in the levels of both nitrate and nitrite in both males and females and in both high and low risk populations. Table 4 shows the effect of social class. Social classes IV and V tend to have lower concentrations of both ions, although the trend across the three social class groupings is not uniform (and is significant only in the low risk populations). Table 5 shows that smoking habits have a consistent effect on salivary concentration of nitrate and nitrite, with smokers always having lower levels than non-smokers. The difference is statistically significant for one group (nitrite secretion in the high risk areas) and highly significant if one pools together the results from high and low risk areas and just considers smoking.

Inverse trend

Table 6 shows the results of a multiple regression analysis which indicates that, even after allowing for the effect of age, social class and smoking, there is still a highly significant difference between the geographically defined high and low risk populations. Contrary to what might have been expected, it is always the high risk population that has the lower concentrations of nitrate and nitrite. Similarly, it

Table 5 Salivary nitrate/nitrite concentrations (nmol per ml) for smokers and non-smokers

	Low risk		High risk		<i>P</i> * (L vs H)		All	
Nitrate								
Current smoker	135.7	(101.2-182.0)	85.8	(66.1-111.3)	<0.05		106.3	(87.2-129.6)
Current non-smoker	172.0	(148.5-199.2)	116.6	(96.9-140.3)	<0.005		145.4	(129.3-163.5)
<i>P</i> †	NS		NS			<0.01		
Nitrite								
Current smoker	87.9	(70.6-109.5)	53.7	(44.3-65.1)	<0.005		67.6	(58.2-78.7)
Current non-smoker	104.6	(92.3-118.6)	73.6	(62.2-87.0)	<0.001		89.8	(81.0-99.6)
<i>P</i> †	NS		<0.005			<0.005		

In the population the stated current smokers were 25% and 33% in the low and high risk areas respectively. Values are geometric means (95% confidence interval).

* P value from two-sided *t*-test comparing low risk with high risk populations within each smoking group.

† P value from two-sided *t*-test comparing current smokers with non-smokers within each population.

is the lower social class grouping which generally have the lowest nitrate and nitrite levels. In both cases, the trend is the inverse of the trend for stomach cancer. If one adds to this the fact that, if anything, smokers have a slightly elevated risk of stomach cancer³³ and also show lower salivary levels, the results presented here all appear to be inconsistent with the notion that nitrate exposure is a risk factor for cancer of the stomach. Indeed, the conclusion could be drawn that, in these circumstances, nitrates or something associated with them exert a protective effect.

The inverse relationship between stomach cancer risk and salivary nitrate and nitrite concentrations raises two distinct problems: why should there be such clear population differences in the levels, and can nitrate exposure still be considered a risk factor for stomach cancer?

We have some evidence bearing on the first question from the dietary questionnaires that the subjects completed when

they gave saliva. The questionnaire asked about the frequency with which the subjects consumed the most common foodstuffs known to contain moderate to high levels of nitrate and nitrite. By converting these frequency responses to daily intake figures (T. Knight and D.F., in preparation), we were able to obtain a crude estimate of the mean population intake levels from dietary sources. These mean levels are shown in Table 7, and are given together with the estimated mean daily intakes of nitrate from drinking water, provided by the Water Research Centre (unpublished results). Overall, it can be seen that there is a substantial difference between the populations, with those from the low risk areas consuming considerably more nitrate from both food and water sources. Although the consumption of exogenous dietary nitrite is significantly higher in the high risk area, this constitutes only a minority component of the total salivary nitrite load. Approximately 5 per cent by weight of exogenous nitrate is converted to nitrite^{28,29} and it is

this that makes the largest contribution to salivary nitrite, rather than dietary nitrite *per se*.

Table 8 shows that there is a close correlation between the estimated amount of nitrate in the diet and the salivary levels of both nitrate and nitrite. The differences between the mean levels in the populations studied can therefore, be attributed, in large part, to the differences of intake shown in Table 7.

Increases of salivary nitrate and nitrite levels with increasing age, similar to those we have found (Table 3), have been reported before³⁴. One explanation could be age-related changes in the active transport mechanism responsible for nitrate uptake into the salivary ducts and, for nitrite, in the density of nitrate-reducing bacteria in the oral cavity. But there is no evidence for such processes.

It is more difficult to explain the slight tendency for salivary levels to be increased in the higher social class groups. One might expect social class differences in dietary habits to result in differential consumption of nitrate-containing foods, particularly vegetables³⁵, but no such trend was apparent in data obtained from our questionnaires, although this could be due to the crudeness of the questionnaire, making it insensitive to narrow differences in diet.

The observation that salivary nitrate levels are lower in smokers than in non-smokers is readily explained by the presence of thiocyanates in cigarette smoke. These are present in substantial amounts and are secreted in the saliva, where they are known to inhibit competitively the uptake of nitrates³⁶.

The differences between our geographically defined populations are also seen in individuals who have fasted on the day of sampling (Table 2). It follows that an individual's salivary nitrate level is not solely determined by recent consumption of food or drink, but is also influenced to some extent by longer-term dietary habits.

Our results in general weigh against the idea that environmental nitrates and nitrites play a major role in determining the risk of gastric cancer in Britain. They should not, however, be taken to imply

Table 6 Coefficients for multiple regression on log nitrate and log nitrite concentrations in refined population (having nothing to eat or drink in the 2 h before sampling)

Model	N	Coefficients (s.d.)				d.f.
		b_1	b_2	b_3	b_4	
Nitrate						
(1)	380	0.18 (0.04)‡	—	—	—	378
(2)	380	0.18 (0.04)‡	0.11 (0.03)‡	—	—	377
(3)	379	0.18 (0.04)‡	0.11 (0.03)‡	-0.10 (0.05)*	—	375
(4)	306	0.15 (0.05)†	0.07 (0.03)*	-0.06 (0.05)	-0.04 (0.02)†	301
Nitrite						
(1)	380	0.17 (0.04)‡	—	—	—	378
(2)	380	0.17 (0.04)‡	0.15 (0.02)‡	—	—	377
(3)	379	0.17 (0.04)‡	0.14 (0.02)‡	-0.08 (0.04)	—	375
(4)	306	0.13 (0.04)‡	0.12 (0.03)‡	-0.05 (0.04)	-0.01 (0.01)	301

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

Models:

$$(1) y = b_0 + b_1 x_1$$

$$(2) y = b_0 + b_1 x_1 + b_2 x_2$$

$$(3) y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3$$

$$(4) y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4$$

where y = log nitrate or nitrite concentration (nmol per ml); x_1 = area (1, high risk; 2, low risk); x_2 = age group (1, 15-34 yr; 2, 35-54 yr; 3, 55-74 yr); x_3 = current smoking status (1, non-smoker; 2, smoker) x_4 = Social Class group (1, I & II; 2, III; 3, IV & V). This model utilizes 306 cases as it was not possible to assign social class groups to the other 74.

Table 7 Daily intake (mg) of nitrate and nitrite from exogenous dietary sources

		Low risk			High risk		P†
	N	Mean		N	Mean		
Intake of nitrate from*:							
Vegetable sources	412	90.29	(84.81–95.77)	386	66.89	(61.45–72.32)	<0.0001
Meat sources	399	2.88	(2.66–3.10)	399	3.49	(3.25–3.73)	<0.001
Other food sources	410	2.79	(2.72–2.86)	411	2.62	(2.55–2.69)	<0.001
Drinking water		24.64			2.21		
Total nitrate intake	384	119.24	(113.56–124.92)	375	74.92	(69.40–80.44)	<0.0001
Nitrite derived from nitrate§	384	5.57	(5.30–5.83)	375	3.50	(3.24–3.76)	<0.0001
Intake of nitrite from:							
Meat sources	399	0.77	(0.71–0.83)	399	1.02	(0.95–1.10)	<0.0001
Other sources	403	0.44	(0.43–0.45)	386	0.43	(0.42–0.44)	NS
Total nitrite intake	384	6.77	(6.49–7.06)	375	4.95	(4.67–5.23)	<0.0001

Intake (arithmetic mean with 95% confidence intervals) was derived from a questionnaire which asked about frequency of consumption of 18 dietary items that constitute the main sources of nitrate and nitrite in the diet. The frequency responses were converted to daily nitrate/nitrite intake values using estimates of their concentrations in these food items in the United Kingdom (from published values) and of average portion sizes (T. Knight and D.F., in preparation and M. Nelson, personal communication). Although limiting the questionnaire to 18 items means that some sources of nitrate/nitrite are not included, these other sources either contain very low levels of the ions or are eaten very rarely. Our estimates of mean daily intake of nitrate (74.9 and 119.2 mg in high and low risk areas respectively) are therefore likely to be slightly lower than the real values. A recent survey of dietary nitrate intake in East Anglia⁴⁷ estimated from diet diary analysis that mean daily intake of nitrate was 60 and 102 mg in areas where the water concentrations of nitrate were similar to our high and low risk regions. Also the NAS report¹² on nitrate exposure in the United States estimated a mean daily intake of 73 mg. Both these surveys are therefore broadly comparable with our own results.

* Vegetable sources asked about were cabbage, cauliflower, lettuce, beetroot, carrot, tomato, green beans and potatoes. The first 3 items provide the majority of the nitrate. Meat sources asked about were bacon, sausage, ham, and other preserved meats—the majority of the nitrite intake was in fact derived from bacon. The other food sources asked about were fresh fruit, bread, breakfast cereals, and spaghetti. Drinking water levels of nitrate were derived from information supplied by the Water Research Centre, Marlow. Their estimates of nitrate concentrations in water (in mg l⁻¹) for our study areas were: Oxford, 15.99; Canterbury, 21.62; Eastbourne, 43.68; Sunderland, 4.47; Hartlepool, 3.23; Bangor and Llandudno, 0.27. We assumed a daily intake of drinking water of one litre and the estimated intake of waterborne nitrate in mg was added to each individual's food intake level to give a total nitrate value. There is no exposure to nitrite in drinking water. N is number completing the relevant section of the questionnaire. In total there were 414 and 422 questionnaires completed in the low and high risk regions respectively. As not all the sections were completed correctly by all the subjects, the number varies slightly from section to section.

† P value for two-tailed t-test comparing mean intake levels for dietary items, low risk populations greater than high risk except for nitrate and nitrite derived from meat.

§ Nitrite derived from nitrate was calculated assuming 6.3% mol (4.7% by weight) of nitrate is converted to nitrite in the saliva²⁸.

that nitrate-related N-nitroso-compound carcinogenesis has no role in the development of gastric tumours; nor should they be taken to exclude the possibility that nitrates may play an important part in countries where the salivary levels are much higher, as in Germany²⁸ and Japan³⁷, where levels 4 to 5 times those in Britain have been reported.

Several factors could contribute to the observed inverse relationship and help to mask a real carcinogenic effect from nitrates *in vivo*. The multistage hypothesis proposed for the aetiology of cancer of the stomach³⁸ requires there to have been degenerative changes in the protective mucosal layer before carcinogens can act on the gastric epithelial cells. It could be

that completely different dietary factors, unrelated to nitrates, are responsible for the development of such conditions, and that these factors may be more important and, in Britain, inversely related to nitrate exposure. There may also be anti-carcinogenic factors (including vitamin C) associated with the vegetables that are the principal source of dietary nitrates.

This might lead one to conclude that it is only meat-derived nitrate and nitrite that constitutes a problem due to the lack of accompanying protective agents. This would be consistent with the observed increase in such intake in the high risk population (Table 7). But waterborne nitrate is similarly unlikely to be associated with such agents, and this more than compensates for the observed differences in meat consumption (Table 7).

It may also be that there is only a narrow range of nitrosatable substances that lead to the production of the N-nitroso compounds acting directly on the human gastric epithelium, and that the amount of these substrates in the diet may be the limiting factor, rather than the amount of nitrate and nitrite ions. It is conceivable, too, that the levels of salivary nitrates and nitrites bear little or no relationship to the levels found in the gastric juice. Salivary nitrites could be selectively absorbed across the gastric epithelium as soon as they reach the stomach, while some other physiological process might determine the gastric

level (ref. 39 and S.R. Tannenbaum, personal communication). Certainly, in the special case of achlorhydria, the decrease of stomach acidity and bacterial colonisation promote the formation of nitrites from nitrates.

The meaning to be attached to the results depends crucially on the validity of the assumption that current levels of exogenous and salivary nitrate and nitrite are relevant to levels of gastric cancer in the recent past. As they cannot have played any direct part in the production of the cancers that determined the mortality rates with which they were compared, we have to assume either that past levels of exposure were proportionately similar or that the ranking of gastric cancer mortality by geographical area and social class will continue to be much as it has been in the recent past for many years to come. In view of the stability of the proportional rates over the past 30 years²⁷, the second assumption seems reasonable, so that the ranking of the current salivary levels may be related to the current ranking of the incidence of the disease. It is possible, however, that the geographical and social class distribution will change in the next 30 years to correspond with the current distribution of salivary nitrate. Also the massive increase in fertilizer usage in recent years⁴ may lead to an effect that will not become apparent for some years yet, as nitrate fertilizers may take 30–40 years to

Nitrates and nitrites as food additives

THE nitrates and nitrites of potassium and sodium are permitted as food additives in the United Kingdom, acting as curing agents or as preservatives (inhibiting, for instance, *Clostridium botulinum*, the bacterium responsible for botulism). On food packaging they are labelled:

- E249 — potassium nitrite
- E250 — sodium nitrite
- E251 — sodium nitrate
- E252 — potassium nitrate

percolate into ground water¹⁰.

Monitoring

It is important, therefore, that trends in mortality should continue to be monitored. Should the overall decline in gastric cancer mortality not continue, or should the geographical pattern of the disease begin to change, our data would have a different implication.

Our data are not unique in failing to show a positive relationship between waterborne nitrate and gastric cancer risk in the United Kingdom. Beresford *et al.*⁴⁰ failed to find one and a lack of relationship has also been reported in the United States⁴¹ and France⁴². Positive relationships have, however, been observed in Italy⁴³, Hungary²², Denmark²¹ and Colombia¹⁸. The inconsistency suggests either that very different conditions pertain in different localities (for example, dependence on well water with very high nitrate levels) or that nitrates play varying roles in different situations.

More direct evidence of the effects of nitrates should be obtainable from the experience of groups of men and women exposed to large amounts of nitrates in the course of their work, in whom other environmental causes of gastric cancer

Table 8 Salivary nitrate and nitrite levels by total daily nitrate intake

Daily nitrate intake (mg)	Nos.	Nitrate	Nitrite
0-39	44	104.3 (75.6-143.8)	68.3 (52.6-88.7)
40-79	117	125.5 (105.0-150.1)	70.7 (60.5-82.7)
80-119	72	144.3 (114.5-182.0)	96.7 (80.1-116.6)
120-159	65	138.8 (108.1-178.2)	87.2 (71.0-107.1)
160+	43	172.0 (128.6-230.1)	98.5 (78.4-123.7)
P (trend)*		<0.05	<0.05

Values are geometric means (95% confidence intervals) for refined populations, excluding those who ate or drank in the 2 h prior to sampling.

* P value for t-test for significance of regression of daily nitrate intake on salivary nitrate or nitrite concentration.

should be the same as those of other residents in the same area. Such groups have been difficult to define, and the available data are inconclusive⁴⁴. More data should, however, be available shortly from a group of fertilizer workers in north-east Britain now under investigation. (S. Al-D., in preparation). □

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1. Brimblecombe, P. *Nature* **298**, 460-462 (1982).
2. Ryder, J.C., Ball, P.R. & Garwood, E.A. *Nature* **311**, 50-53 (1984).
3. Corre, W.J. & Breimer, T. *Literature Survey 39* (Centre for Agricultural Publishing and Documentation, Wageningen, 1979).
4. Royal Commission on Environmental Pollution, seventh report: *Agriculture and Pollution* (Cmd 7644, HMSO, London, 1979).
5. White, R.J. *Aqua* **2**, 51-57 (1983).
6. Young, C.P. & Gray, E.M. *Water Research Centre Technical Report TR 69* (Water Research Centre, Medmenham, 1978).
7. Statutory Instruments: *The Preservatives in Food (Amendment) Regulations* (HMSO, London, 1962, 1979 & 1982).
8. *The Nitrogen Cycle of the United Kingdom* (Royal Society, London, 1983).
9. Tannenbaum, S.R., Weisman, M. & Fett, D. *Food Cosmet. Toxicol.* **14**, 549-552 (1976).
10. Fraser, P. & Chilvers, C. *Science Total Envir.* **18**, 103-116 (1981).
11. Magee, P.N., Montesano, R. & Preussmann, R. *ACS Monogr.* **173**, 491-625 (1976).
12. US National Research Council. *The Health Effects of Nitrate, Nitrite, and N-nitroso compounds* (National Academy Press, Washington, 1981).
13. Mirvish, S.S. *J. natn. Cancer Inst.* **71**, 631-647 (1983).
14. Mirvish, S.S. *Tox. appl. Pharmac.* **31**, 325-351 (1975).
15. Sugimura, T. & Kawachi, T. in *Gastrointestinal Tract Cancer* (eds Lipkin, M. & Good, R.) 327-340 (Sloan Kettering

- Intitute, New York, 1978).
16. Hartman, P.E. *Envir. Mutag.* **5**, 111-121 (1983).
17. Hill, M.J., Hawksworth, G.M. & Tattersall, G. *Br. J. Cancer* **28**, 562-567 (1973).
18. Cuello, C. *et al. J. natn. Cancer Inst.* **57**, 1015-1020 (1976).
19. Zaldivar, R. *Zbl. Bakt. Hyg. I. Abt. Orig. B.* **164**, 193-217 (1977).
20. Haenszel, W., Kurihara, M., Locke, F.B., Shimizu, K. & Segi, J. *J. natn. Cancer Inst.* **56**, 265-274 (1976).
21. Jensen, O.M. *Ecotox. Envir. Safety* **6**, 258-267 (1982).
22. Juhasz, L., Hill, M.J. & Nagy, G. *IARC Scientific Publ.* **31**, 619-623 (IARC, Lyon, 1980).
23. Amadori, D. *et al. Tumori* **66**, 145-152 (1980).
24. Fraser, P. in *Interpretation of Negative Epidemiological Evidence for Carcinogenesis* (eds Wald, N. & Doll, R.) (IARC and Green College, Lyon and Oxford, in the press).
25. Armijo, R. *et al. Int. J. Epidemiol.* **10**, 57-62 (1981).
26. Davies, J.M. *Br. J. Cancer* **41**, 438-445 (1980).
27. Registrar-General's *Statistical Reviews of England and Wales* (HMSO London, 1950-1973); *Office of Population Censuses and Surveys: Mortality Statistics — Area England and Wales Series DH5 nos. 1-8* (HMSO, London, 1974-1981).
28. Spiegelhalter, B., Eisenbrand, G. & Preussman, R. *Food Cosmet. Toxicol.* **14**, 545-548 (1976).
29. Stephany, R.W. & Schuller, P.L. *Oncology* **37**, 203-210 (1980).
30. *Mortality Statistics — Area 1969-1973* (Office of Population Censuses & Surveys, HMSO, London, 1981).
31. Al-Dabbagh, S.A. thesis. Oxford Univ.
32. Phizackerley, P.J.R. & Al-Dabbagh, S.A. *Analyt. Biochem.*

- 131**, 242-245 (1983).
33. Doll, R. & Peto, R. *Br. med. J.* **2**, 1525-1536 (1976).
34. Eisenbrand, G., Spiegelhalter, B. & Preussman, R. *Oncology* **37**, 227-231 (1980).
35. Nelson, M. *MRC Environmental Epidemiology Unit Scientific Report No. 4*, 16-21 (1984).
36. Hartmann, P.E. *Chemical Mutagens* Vol. 7 (eds de Serres, J.J. & Magee, P.N.) 211-294 (Plenum, New York, 1981).
37. Ishiwata, H., Boriboon, P., Harada, M., Tanimura, A. & Ishidate, J. *J. Food Hyg. Soc.* **16-2**, 83-98 (1975).
38. Correa, P., Haenszel, W., Cuello, C., Tannenbaum, S. & Archer, M. *Lancet* **ii**, 58-60 (1975).
39. Green, L., Ralt, D. & Tannenbaum, S.R. in *Human Nutrition, Current Issues and Controversies*, 87-140 (MTP Press, Lancaster, 1982).
40. Beresford, S.A. *Int. J. Epidemiol.* (in the press).
41. Geleperin, A., Moses, V.J. & Fox, G. *Illinois med. J.* **149**, 251-253 (1976).
42. Vincent, P., Dubois, G. & Leclerc, H. *Rev. Epidem. Sante. Publique* **31**, 199-207 (1983).
43. Gilli, G., Correo, G. & Favilli, S. *Sci. Total Envir.* **34**, 35-48 (1984).
44. Fraser, P., Chilvers, C. & Goldblatt, P. *Br. J. ind. Med.* **39**, 322-329 (1982).
45. *Mortality Statistics — Area (1981 No.DH5-8)* (Office of Population Censuses and Surveys, HMSO, London, 1983).
46. *Classification of Occupations: 1980* (Office of Population Censuses and Surveys, HMSO, London, 1980).
47. Chilvers, C. *et al. Int. J. Epidemiol.* **13**, 324-331 (1984).

Hopes grow for supersymmetry

from John Ellis

High-energy collisions between protons and antiprotons produce strange events in which momentum fails to balance. Missing momentum may be carried by photinos, super-partners of the photon.

THE proton antiproton ($p\bar{p}$) collider at CERN, the European organization for nuclear research, is the first particle accelerator to explore physics in the energy range up to about 100 GeV. Therefore it has had a golden opportunity to detect new particles and observe other new phenomena. Already the periods of collider operation in 1982 and 1983 have provided a rich crop of discoveries: those of the $W^\pm$ and Z^0 intermediate vector bosons announced in 1983 and possible evidence for the top quark published in 1984. Along with the new particles, the 1983 experiments produced evidence, albeit inconclusive, of events that are not easily explicable by established theories, such as quantum chromodynamics (QCD) or the Weinberg-Salam model of the electroweak interactions. Just as the existence of the neutrino was proposed by Fermi to account for the momentum which seems to be missing from weak interactions, so today's physicists are postulating that similar anomalies in the CERN results can be explained by 'sparticles' — particles whose existence is predicted by theories of supersymmetry. (For a very readable introduction to the ideas and terminology in this field, see ref.1).

The unexpected events seen in 1983 include many radiative decays of Z^0 into a lepton pair plus a photon, a possible bump in dijet masses around 150 GeV, and several categories of events with missing transverse momentum. Among the latter are lepton plus jet events², single jets of particles, one event with three jets, and one or two events containing electromagnetic showers due to one or more photons³, all with unbalanced transverse momentum. The statistical significance was not compelling for any of these categories of events, but this has not prevented physicists from speculating energetically about their possible interpretations.

Advocates of composite structures for quarks, leptons and gauge bosons have been encouraged by many of the possible new phenomena, while advocates of supersymmetry have been particularly intrigued by the events with missing transverse momentum. The only known sources of missing transverse momentum are neutrinos, as known, for example, from decays of a W to an electron plus neutrino or to a muon plus neutrino ($W \rightarrow e\nu$ or $\mu\nu$). The lepton plus jet events with missing transverse momentum could have been due to the production by higher-order QCD pro-

cesses of W s with associated jets. This, however, is not a possible interpretation for the 'monojet' events containing a single hadronic jet with missing transverse momentum. Nor are these events easy to explain by other 'conventional' physics, such as the decay of a W to a tau plus neutrino ($W \rightarrow \tau\nu$) followed by the decay of the tau to hadrons plus neutrino, or the pair-production of heavy flavours followed by their semileptonic decay (charm or bottom $\rightarrow e$ or $\mu + \nu$ + hadrons). An alternative mechanism for producing neutrinos is the production of jets with Z^0 bosons decaying into $\nu\bar{\nu}$ pairs. The monojets observed in 1983 seem to occur at a rate much higher than expected from QCD calculations of Z^0 production, and no analogous events, containing jets plus a Z^0 decaying either into charged lepton pairs or into hadrons were seen. While by no means conclusive, these facts argue against neutrinos as the source of missing transverse momentum and suggest the presence of some new species of neutral, weakly-interacting and stable (or quasi-stable) particle.

Supersymmetry provides a natural theoretical home for such a particle. In most such theories, heavy supersymmetric particles decay into lighter ones, and the lightest of all these sparticles would be absolutely stable, should be electrically neutral and should have no strong interactions. If it did it would bind to ordinary matter and give too large an abundance of anomalously heavy isotopes. The most obvious candidate for this sparticle seems to be the photino, the spin $\frac{1}{2}$ partner of the photon, but other possibilities are also open. One could expect squarks — the spin 0 partners of the quarks — to decay into quark plus photino or gluino, and gluinos — the spin $\frac{1}{2}$ partners of the gluons — to decay into quark, antiquark and photino. Theoretical ideas about the masses of sparticles are quite vague but can play a useful role in fixing the weak interaction scale if they weigh less than about 1 TeV. Within this range, the values of their masses are rather model-dependent⁴.

Qualitatively, supersymmetry certainly suggests the possible observation of events with missing momentum carried away by photinos. Quantitatively, supersymmetric particle couplings are completely specified, so one can reliably calculate the cross-sections for producing them, as functions of their unknown masses. One can therefore use the relatively small number of missing monojet events observed in 1983

to give lower bounds on the possible masses of squarks and gluinos. More radically, one could try to interpret the observed monojet events in terms of squark or gluino production and decay⁵⁻⁸. Since these supersymmetric particles are strongly interacting they are likely to have the largest production cross-sections in $p\bar{p}$ collisions. In interpreting the 1983 data, care must be taken to implement correctly the conditions under which the apparatus was triggered and the data were analysed. Hadrons emerging from the collision point within about one steradian of solid angle were coalesced into jets, and only events containing at least one jet with a transverse energy above about 25 GeV were recorded. Additional 'jets' with transverse energies less than about 12 GeV were not thought to be measured very reliably, and so were put on one side. In fact, all the 1983 'monojet' events contained one or more such 'minijets'.

Monojets

The way in which the 1983 data were collected induced a 'trigger bias' which means that the pair-production of either squarks or gluinos yielded predominantly monojet configurations, at least as long as the squark or gluino mass was less than about 40 GeV. The trigger bias favours events where, for example, one squark decays into an energetic quark and a soft photino, and vice versa for the other squark, or towards configurations where the two quark jets coalesce into one as seen by the apparatus. Were it not for the bias one would have expected each quark to give a quark jet, making two jets per event, and each gluino to give both a quark and an antiquark, making four jets per event. The relatively small number monojet events observed allowed the conclusion^{5,6} that squarks and gluinos could not have masses much less than 40 GeV, whereas the previous lower limits were about 20 GeV for squark mass and 4 GeV for gluino mass. More excitingly, they raised questions of whether the observed monojets could be interpreted in terms of either squarks or gluinos with masses around 40 GeV; whether there could be other supersymmetric interpretations of the monojet events; how one could discriminate between the different interpretations; and what experiments could help resolve these issues?

The 1983 data provided two possible, but statistically insignificant, reasons for preferring^{6,9} the squark pair-production inter-

pretation over gluino pair-production. One is the hardness of the spectrum of monojet transverse energies, and of missing transverse momenta. While not trivial to explain with either squark or gluino pairs, squarks are favoured because of their harder, two-body, squark to quark decay spectrum. The other reason is the thinness of the observed monojets, which seem to contain fewer charged particles with lower invariant masses than conventional QCD jets. Neither squarks nor gluinos tend to give thin enough jets, but gluino jets are thicker on average and hence disfavoured.

A third possible interpretation⁷ of the monojets as a signature for supersymmetry predates their discovery. The idea is that if gluinos only weigh a few GeV, a significant number of them would be contained intrinsically as partons in the proton and antiproton, which could fuse with quarks and antiquarks in the other beam to form squarks weighing 80 to 100 GeV. Some of the squarks so produced would decay into a quark plus a photino, producing monojet events. Such monojets would exhibit a Jacobian peak in transverse energy, analogous to that seen for electrons from $W \rightarrow \bar{\nu} \nu$ decay.

Dijets

However, while this light gluino-heavy squark mechanism sidesteps trigger bias as an explanation of the sighting of monojets, there are some potential difficulties which have to be resolved. For every decay of squark to quark plus photino there should be many decays of squark to quark plus gluino, which would give two jet events with missing transverse momentum aligned almost parallel to the softer jet. Another potentially copious source of such 'coplanar' dijets is pair-production and decay of the light gluinos. A more theoretical difficulty is that models with such light gluinos tend to contain stable photinos weighing less than one GeV, which would give more dark matter in the Universe than is consistent with astronomical considerations. Since none of these arguments conclusively favours one interpretation over the other, and since the 1983 data may have nothing to do with supersymmetry, it is important to search for other signatures which could support one of the supersymmetrical interpretations. One obvious candidate is the dijet events¹⁰. If squarks or gluinos have masses of the order of 40 GeV, dijet events would be expected at about half the rate of monojet events. Squark or gluino pairs usually give configurations in which the decay jets are not coplanar, in contrast to the light gluino-heavy squark mechanism. In addition, one would expect there to be more subtle differences between the energy and angle distributions of dijet events from squarks and gluinos; and the differences might ultimately help distinguish between the squark and gluino interpretations. Finally, how about trijet events with missing transverse momentum, one of which

was observed among the 1983 data? These emerge naturally from gluino pair-production, can also arise from higher order corrections to squark pair-production, but are less natural in a light gluino/heavy squark mechanism.

So far we have only discussed the 1983 data, but first glimpses of the 1984 data were becoming available late last year, for example in a talk by Carlo Rubbia at the Rutherford Appleton Laboratory Theoretical Physics meeting last December. The UA1 collaboration, which he heads, had seen no new candidates for radiative decays of the Z^0 , nor had there been an excess of lepton plus jet events with missing transverse momentum. Monojets, however, were rolling in at a rate comparable to the previous data, suggesting that they are not just a statistical fluke. Most interestingly, a few candidate dijet events with missing transverse momentum had been found. At least some of these are not of the coplanar type, and therefore the light gluino/heavy squark interpretation of the monojets is perhaps disfavoured.

What if we throw caution to the winds, and assume⁹ that squarks with a mass around 40 GeV have been observed, while gluinos have masses above 40 GeV? Supersymmetric grand unified theories enable one to calculate most of the supersymmetric particle masses in terms of just three parameters: the bare spin 0 sparticle mass, the bare spin $\frac{1}{2}$ gaugino mass, and a ratio of Higgs vacuum expectation values. The choice of these parameters is now tightly constrained, along with the possible masses of other, unobserved, sparticles. For example, sleptons, the spin 0 partners of the charged leptons, would then be expected to have masses of 20–30 GeV, the photino mass would be 5–8 GeV and gluinos should have masses less than about 60 GeV. Gluino production should therefore be detectable at the CERN $p\bar{p}$ collider, while slepton pair-production could be seen with positron-electron (e^+e^-) machines at energies slightly higher than those attained by PETRA. An interesting process to search for with present e^+e^- machines, is the annihilation of an e^+e^- to an invisible pair of photinos, tagged by a bremsstrahlung photon. Such 'monophoton' events have already been looked for by the MAC collaboration at PEP, which even has one doubtful candidate, taken as an upper limit on the cross-section. A new experiment with improved sensitivity, called ASP, has been prepared in record time and is now taking data at the Stanford Linear Accelerator. If the squark mass is around 40 GeV, monophoton events should occur at a rate very close to the limit set by MAC. They would not be expected by the other interpretations of the $p\bar{p}$ collider monojet events, and so could help distinguish between the different models.

If the sleptons do indeed weigh less than about 40 GeV, supersymmetric decays of the $W^\pm$ and Z^0 into slepton pairs^{11–13} should be observable at the CERN $p\bar{p}$ col-

lider. The most distinctive signature may be for the decay of Z^0 into selectron and anti-selectron, which in turn decays into an e^+e^- pair with large transverse momentum and about 20 GeV missing transverse momentum carried off by a pair of photinos. If the selectron weighs less than about 30 GeV, one would expect one such event for every ten or so conventional decays of the Z^0 into e^+e^- . With more than 20 such Z^0 decays in their 1984 data, the UA1 collaboration should soon be able to discriminate between different interpretations of the monojet events.

In the past few years, we have become used to hearing negative results from searches for supersymmetric particles. The $p\bar{p}$ collider monojet events are the first possibly positive results, though their interpretation is still far from clear. Confirmation of some interpretations of the data will require at the very least the discovery of other missing transverse momentum events with different topologies. Possible candidates include dijet or e^+e^- events with missing transverse momentum in $p\bar{p}$ collisions, or monophoton events in e^+e^- collisions.

Patience

The present situation is reminiscent of the discovery of neutral currents at CERN in 1973. Then it was the combination of hadronic neutral current events (which were relatively 'dirty' because of worrisome backgrounds despite good statistics) and leptonic neutral current events, (which were relatively 'clean' with low and well-understood backgrounds but limited to one or two events) which was crucial in convincing physicists of the reality of neutral currents. Perhaps monojet and dijet events will play the role of hadronic neutral current events, and e^+e^- or monophoton events the role of leptonic neutral current events in establishing supersymmetry. Will the full analysis of the 1984 collider data reveal supersymmetry, or will we have to be more patient? Perhaps the present data have nothing to do with supersymmetry. It could even be that Nature knows nothing of supersymmetry — but that would seem to be a waste of a beautiful theory. □

1. Llewellyn Smith, C.H. *Nature* **312**, 588 (1984).
2. Bagnaia, P. et al. *Phys. Lett.* **139B**, 105 (1984).
3. Arnison, G. et al. *Phys. Lett.* **139B**, 115 (1984).
4. *Supersymmetry versus Experiment* (ed. Nanopoulos, D.V. & Savoy-Navarro, A.) *Phys. Rep.* **105C**, 1 (1984).
5. Reya, E. & Roy, D.P. *Phys. Lett.* **141B**, 442 (1984); *Phys. Rev. Lett.* **52**, 881 (1984).
6. Ellis, J. & Kowalski, H. *Phys. Lett.* **142B**, 441 (1984); *Nuclear Phys.* **B246**, 189 (1984).
7. Herrero, M.J., Ibañez, L.E., López, & Yndurain, F.J. *Phys. Lett.* **132B**, 199 (1984); **145B**, 430 (1984).
8. Barger, V., Hagiwara, K., Woodside, J. & Keung, W.Y. *Phys. Rev. Lett.* **53**, 641 (1984); U. Wisconsin preprint MAD/PH/197 (1984).
9. Ellis, J. & Sher, M. *Phys. Lett.* **148B**, 309 (1984).
10. Ellis, J. & Kowalski, H. preprint CERN-TH.4072 (1984).
11. Cabibbo, N., Maiani, L. & Petrarca, S. *Phys. Lett.* **137B**, 195 (1983).
12. Barnett, R.M., Haber, H.E. & Lackner, K. *Phys. Rev. Lett.* **51**, 176 (1983).
13. Baer, H., Ellis, J., Nanopoulos, D.V. & Tata, X. preprint CERN-TH.4059 (1984).

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Astronomy

Quasar birthpangs observed?

from C. Martin Gaskell

BETWEEN 5–10 per cent of high-redshift quasars show broad absorption lines arising from gas being expelled at speeds of up to 10 per cent of the speed of light. A recent study published in *Astrophysical Journal* **282**, 33; 1984 by Cyril Hazard and collaborators leads to the suggestion that these so-called broad absorption line (BAL) quasars are actually quasars turning on for the first time.

Early studies showed that quasars are more common the further back in time one looks (that is, at higher redshifts). This produced confident predictions that surveys down to faint optical magnitudes and low radio-flux levels would turn up many high-redshift quasars. However, despite the big advances over the past decade in radio and optical telescopes, and in particular in low-light level detectors, the quasar redshift record has only crept from 3.53 in 1974 to 3.78 in 1982. This lack of progress should be contrasted with the situation for more normal (non-quasar) galaxies over the same period, where the same advances in instrumentation have pushed the redshift record from 0.46 to 1.82.

Where are the quasars with a redshift (z) of greater than 3.8? As search techniques have been refined in recent years, it has become increasingly apparent that there is a real deficit of quasars with z of greater than 3.2, and this has led many people to suggest that by z of say 3.5 we have already reached the epoch of quasar formation (and perhaps the epoch of galaxy formation as well). If this is true, then we might well expect high-redshift quasars to show spectroscopic signs of their youth.

One of the techniques being developed to search the sky for quasars (and other interesting objects) is objective-prism spectroscopy using the UK Schmidt telescope in Australia. The prism in front of the 48-inch Schmidt telescope produces millions of tiny spectra of extremely faint objects over a $6^\circ \times 6^\circ$ field of view. Hazard, one of the pioneers of this technique, and his collaborators report the discovery of nine new broad absorption-line quasars on objective-prism plates, expressing particular interest in their redshift distribution. Much of their paper is spent discussing the difficulties of recognizing BAL quasars on the UK Schmidt objective-prism plates. Normal quasars can be recognized relatively easily by their strong emission lines but the identification of quasars with broad absorption lines is much harder and in some cases almost impossible without follow-up spectroscopy on a large telescope. Their detectability is a strong function of their distance, because their redshifts govern what features fall within the observed wavelength region.

Hazard *et al.* expect to be able to detect BAL quasars in the redshift range of about 1.4–3, with the range of about 2.1–2.3 being particularly favoured. Four of their nine BAL quasars fall in the favoured redshift range, but the five that lie outside this range all have higher redshifts. This is surprising, since one finds more non-BAL quasars in the redshift range of 1.4–2 than 2.4–3. It seems, therefore, that the fraction of quasars with broad absorption lines rises steeply at high redshifts.

This discovery is important. It leads Hazard *et al.* to suggest that BAL quasars are young quasars and that the broad absorption troughs are the result of some violent ejection during the first onset of quasar activity. In the course of time the BAL quasar will evolve into a more normal

quasar. This contrasts with earlier suggestions that BAL quasars are either a special kind of quasar or, perhaps, are normal quasars viewed from a special angle. Whatever the cause of BAL quasars, the rise in the percentage of quasars showing broad absorption lines as redshift 3.5 is approached is strong support for 'something' having happened about that time.

Hazard *et al.* also point out that if many quasars pass through the broad absorption line phase, with heavy elements being rejected at high velocities, there could be a considerable influence on the pollution and dynamics of the intergalactic medium and the formation of galaxies. Full confirmation of these ideas will require more extensive studies of the frequency of BAL quasars among the quasar population as a whole and more follow-up spectroscopy of quasars found to have peculiar spectra on the UK Schmidt prism plates. □

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Geochemistry

Multistage accretion and core formation of the Earth

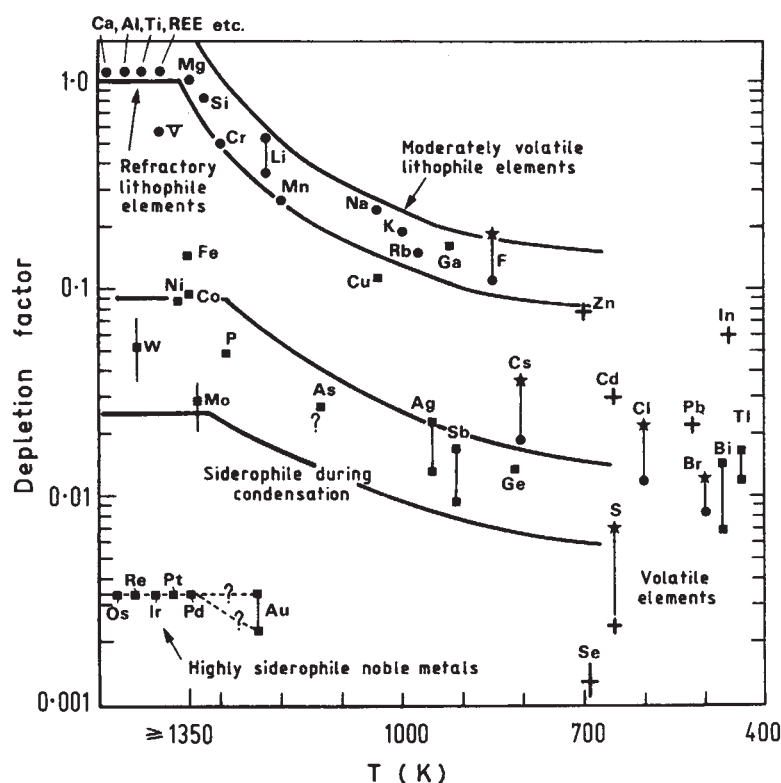
from Shen-su Sun

THERE are several competing hypotheses to explain the Earth's accretion from the solar nebula and the subsequent formation of its metal core within a period of 100 million years, about 4.6 thousand million years ago. Arguments are based on models of the development of the Solar System, studies of meteorites, experiments carried out under high temperature and pressure, and our knowledge of elemental abundances in the Earth's mantle. Specific processes of accretion and core formation have left their finger prints on the abundance patterns of elements with different affinities for silicates, metallic iron and sulphides. The strengths or weaknesses of various hypotheses can therefore often be exposed when accurate abundance data for certain critical elements become available. A good example is provided by a recent study by Newsom and Palme of the abundances of molybdenum (Mo) and tungsten (W) in the Earth's mantle¹, which lends strong support to multi-stage accretion models and severely limits the role played by metal and sulphide during the final stage of accretion.

As a result of the accretion processes, many elements are depleted in the Earth's mantle relative to their abundance in type 1 carbonaceous chondrites (C1)—believed to represent the most primitive Solar-System material. The general sequence of depletion factors for lithophile elements in the Earth's mantle relative to chondrites (see figure) corresponds approximately to

the predicted sequence of condensation of elements and phases from a cooling gas of solar composition. The regular distribution patterns suggest that the terrestrial depletion is related to the character of the source material within the Solar System and/or is a consequence of a selective condensation/volatilization process which occurred during Earth's accretion. Depletion of the siderophile elements in the mantle reflects their preferential entry into the core (about one third of the Earth's mass) but the degree of loss of individual elements during accretion varies according to their volatility. The highly siderophile elements (platinum group elements, Au) are depleted in the mantle by a factor of about 300 but maintain chondrite ratios relative to each other. A surprising feature of the figure is that the abundances of siderophile elements in the mantle are much greater than would be predicted by partition coefficients¹ between iron and silicates expected to occur in the mantle. A multi-stage process of accretion and core formation is therefore required^{3–5}.

One viable model of that type has three main stages. In the first, there is accretion of 85–90 per cent of the Earth from reduced chondritic components with simultaneous formation of the iron core; all highly siderophile elements and most moderately siderophile elements are carried into the core, with minor retention of Ni, Co and FeO in the silicate mantle. In the second stage, after initial core formation,



Elemental depletion factors versus solar condensation temperature for the Earth's primitive mantle. Depletion factor is the ratio of the abundance of an element in the primitive mantle to that in type 1 carbonaceous chondrites relative to Mg. Square symbols denote a siderophile, circles a lithophile, and plus signs a chalcophile during solar condensation. Stars represent possible upper limits. Modified from Fig. 3 of ref. 2 to include new data on W and Mo from ref. 1.

10–15 per cent of more oxidized chondritic components are added. This may result in cessation of the main core-forming event, because the accreting material contains much less metal and the mantle becomes more oxidized. The oxidized components could be of C1 chondrite type, thus accounting for the abundances of siderophile and volatile elements in the mantle. A continuous change of the oxidation character of the source material from the first stage to the second seems likely. Extraction of a very small amount (much less than one per cent) of sulphide-rich iron from the partially molten mantle into the core could severely deplete highly siderophile elements whereas moderately siderophile elements would be depleted to different extents. Ni, Co, Cu and Ga remain in the silicate mantle. Finally, in stage three, accretion of about one per cent of chondritic material brings in highly siderophile elements and establishes the low abundances that are now observed in the mantle.

Newsom and Palme point out that because Mo has a stronger affinity than W for iron (siderophile) and sulphur (chalcophile), the depletion of Mo relative to W provides a sensitive indicator of the second-stage process. Deviations within a factor of two of the newly estimated Mo/W ratio for the Earth's mantle from the expected chondritic value for the bulk Earth would limit metal or sulphide segregation from the mantle to the core during stage two to pro-

bably less than 0.1 per cent, otherwise stronger depletion of Mo relative to W, Co and Ni would be observed. Similar normalized abundances of Cu (highly chalcophile) to Co (moderately chalcophile) lend support to this conclusion.

It is worthwhile pointing out, however,

that these new Mo and W data do not preclude the possibility that sulphide might have played an important role during the first stage of core formation. Critical evaluation of the suggestion⁶ that sulphur is a major light element in the core, which has a density about 10 per cent lower than pure Fe, requires independent evidence. Ringwood^{5,7} has pointed out that oxygen is the most abundant element in the Earth and it is highly soluble in molten iron under the high pressures and temperatures that accompany core formation. It seems inevitable that during the Earth's accretion the iron core incorporated about ten per cent of oxygen. The lithophile element-depletion pattern illustrated in the figure, however, suggests that sulphur was severely depleted in the Earth-forming material and/or lost by volatilization during Earth's accretion. On the basis of the depletion pattern, it is feasible that the Earth's core might contain about two per cent sulphur. The full significance of the detailed abundance patterns of moderately siderophile and volatile elements shown in the figure remain to be explored. Their application to differentiation of the Earth's mantle during the accretion and core formation should provide us with a much better appreciation of the history of the Earth. □

1. Newsom, H.E. & Palme, H. *Earth planet. Sci. Lett.* **69**, 354 (1984).
2. Sun, S.-S. *Geochim. cosmochim. Acta* **46**, 179 (1982).
3. Wänke, H. *Phil. Trans. R. Soc. A* **303**, 287 (1981).
4. Morgan, J.W., Wandless, G.A., Petrie, R.K. & Irving, A.J. *Tectonophysics* **75**, 47 (1981).
5. Ringwood, A.E. *Proc. R. Soc. A* **395**, 1 (1984).
6. Rama Murthy, V. & Hall, H.T. *Phys. Earth Planet. Inter.* **2**, 276 (1970).
7. Ringwood, A.E. *Geochim. J.* **11**, 111 (1977).

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Ecology

How and why eroded ecosystems should be restored

from Jared Diamond

RESTORATION ecology is emerging as an applied science related to the basic science of ecology in the same way that medicine relates to molecular and cell biology. It aims to repair, create, or recreate ecosystems and ecological communities. Typical goals include converting eroded areas into productive land, restoring fish production in overfished or polluted lakes, and recreating vanished communities. To lay the conceptual foundations of restoration ecology, it will be necessary to identify reasonable goals, to define the minimum scientific knowledge necessary to achieve those goals, and to exploit the potential spin-offs from restoration ecology to 'pure' ecology. These three aims were explored in a symposium organized by the University of Wisconsin Arboretum and

held in Madison on 11–12 October 1984. Founded just after the dustbowl disaster of the 1920s, the Arboretum was a most appropriate host because of its role in recreating samples of Wisconsin's vanished prairies and forests and its experiments on the use of fire in prairie management.

The issue of goals came sharply into focus over the repeated mention of the words 'natural' and 'self-sustaining', and over the contentious question of whether restoration ecologists may use only native species. If restoration ecology had the single goal of restoring defined natural communities, the use of exotic species would obviously be anathema. Reality is more complex, for at least five reasons.

First, no community on Earth has escaped the direct or indirect effects of

man, so which is the 'natural community' that one would seek to restore? For instance, the stated mandate of the US National Park Service is "to preserve the parks for posterity in essentially their natural state". Does that mean the exact state first seen by Europeans, or the state after correction for effects of successional processes then operating, or corrected for prehistoric effects of Amerindians? Should one merely prevent man-made fires, or should one deliberately simulate fires set by Amerindians or lightning? Such seemingly arbitrary choices can have major consequences; for example, should the restored park be closed forest, open forest or prairie. Second, even if the goal of a natural community could be specified, it is often unattainable because important native species have been exterminated and introduced species have become firmly established. Third, the resulting semi-natural community may not be self-sustaining without continual management to compensate for tiny population sizes, severed nutrient inputs and severed migration routes (see *Nature News and Views* 289, 350; 1981). Fourth, increments of 'naturalness' cost money and time that might better be spent elsewhere: should one make a costly attempt to eradicate the last few exotic plant species from a restored woodland, or spend the money on restoring another woodland? Finally, there are many cases where a restoration project has legitimate goals unrelated to naturalness, such as preventing erosion or restoring timber and fisheries production. Thus, the goals of restoration ecology are much broader than simply restoring natural communities. Instead there is a spectrum of goals, which is responsive to a spectrum of needs and constraints; and each goal requires application of ecological knowledge.

What ecological knowledge is necessary? A seemingly prudent answer would be to demand detailed studies of each species, their interactions with each other and with the environment. This may be appropriate when ample money and time are available, but the usual challenge of restoration ecology is to make do with much less knowledge. Rainforests, for instance, consist of thousands of plant and animal species, for most of which the details of reproduction are unknown. More generally, restoration ecology is a crisis discipline operating under short deadlines and fixed budgets where the central methodological question is the ecological information that needs to be acquired in order to achieve the particular goal. Much discussion at the symposium centred around the tension created by adapting the methods and values of a pure science to the needs of an applied science. For example, the reclamation of mined lands is usually accomplished by irrigation and fertilization of the land, followed by dispersal of seed by machine. An alternative approach is to use seed-eating mammals to disperse the seed, but this requires knowledge of the seed types

most likely to be dispersed by animals. Rather than solve this question for each granivore species, James MacMahon (Utah State University) showed how the information could be achieved cheaply and elegantly for whole classes of species by setting out dishes of seeds in the desert by night or by day, inside or outside cages. Desert ants, birds and rodents all turn out to select seeds by their caloric or soluble carbohydrate content.

Amidst these discussions of practical problems, the symposium also examined two ways in which restoration ecology could make unique contributions to basic ecology. First, Anthony Bradshaw (University of Liverpool) pointed out that the acid test of a presumed understanding of an ecosystem, as of a clock or enzyme reaction chain, is whether it can be reassembled in working order from its individual components. He illustrated his point with a

dramatic photograph of a 'restored' British waste dump, still bare of vegetation because its need for nitrogen had been overlooked. Restoration ecology's synthetic approach thus complements the reductionist approach of academic ecology in understanding ecosystems. Second, although the controlled experiment is as powerful a tool in ecology as in other sciences, most conceivable manipulations of ecosystems are illegal, immoral, or impractical (see *Nature News and Views* 304, 586; 1983) and ecological field experiments are doomed to operate for short times on tiny areas. Only in restoration projects can ecologists exterminate or introduce species over large areas and plough up whole landscapes. Thus, restoration ecology vastly expands the scale of experimental ecology. □

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Genetic engineering

Better enzymes by design

from E.T. Kaiser

A BURGEONING activity among those interested in the mechanism of enzyme action has become the redesign of active sites, as well as of structural regions, through the use of the technique of site-directed mutagenesis. Because this technique permits the systematic replacement of particular amino-acid residues by other residues of any characteristic the 'designer' wishes to introduce, it is rapidly replacing the technique of chemical conversion. The latter technique has had some success in converting catalytically crucial nucleophilic residues in an enzyme into other potentially reactive nucleophilic groups^{1,2}, but is limited by the requirement for reactions to be targeted specifically to one residue in a protein molecule and by the need to carry out conversion reactions under mild conditions, typically at ambient temperatures and at pH values near neutrality. No such constraints are placed upon site-directed mutagenesis as long as cloned DNA for the enzyme in question can be obtained. The technique is already showing its paces.

So far, the genetic engineering of enzyme active sites has focused on catalytic species involved in acyl transfer reactions such as the enzyme β -lactamase. Typically, a short synthetic DNA sequence encompassing the desired mutation is used either to prime production of double-stranded cDNA prior to cloning or to replace a gene fragment excised from a DNA duplex. The active-site serine in β -lactamase has in this way been replaced by a cysteine residue³ and by a threonine residue⁴. Although the former mutant catalyses the hydrolysis of penicillin with a K_m value similar to that measured for the parent enzyme, the k_{cat} of the mutant enzyme is approximately 100 times smaller. With the cephalosporin,

nitrocefin, as the substrate, the K_m of thiol- β -lactamase is increased more than 10-fold, and the k_{cat} is at least comparable to that of the parent enzyme⁵. When the active-site serine is converted to a threonine residue, on the other hand, the enzyme loses its catalytic activity towards penicillin⁴.

Although the number of enzymes catalysing acyl transfer in which active-site nucleophiles have been converted into new potentially reactive nucleophiles is still rather small, the examples studied to date show low activity towards all but the more highly-activated substrates. In most acyl transfer reactions the catalytic pathway involves formation and decomposition of tetrahedral intermediates (with the possible exception of cases with very good leaving groups) and requires a series of rapid proton transfers. The replacement of the nucleophilic residue at the active site of a proteolytic enzyme by a new nucleophile in a mutant form may, in a particular molecular environment, hinder some proton transfer steps and cause a bottleneck. If this explanation is correct, then generation of the effective new active-site nucleophiles in acyl transfer enzymes may require concomitant alteration of the nearby environment by additional mutations.

Recently, the possibility has been explored that alteration of active-site nucleophiles may have less deleterious effects in enzymes catalysing other types of group transfer reactions, where rapid proton transfers may not be a requirement for effective catalysis. In my laboratory, we find that conversion of the active-site serine to cysteine in alkaline phosphatase of *Escherichia coli* does not alter the enzyme's high degree of catalytic activity toward a

number of phosphate monoesters. This may be due to a less stringent requirement for rapid proton transfers in the phosphoryl transfer process than is usual in acyl transfer reactions. If so, it may provide a means of predicting species that are effective at catalysing group transfer reactions.

Several other experiments have tested the effects of altering non-nucleophilic residues in enzymes, and have produced intriguing results. In rat trypsin, glycine residues 216 and 226 have been replaced by alanines (C.S. Craik *et al.*, personal communication). As expected from analysis by computer graphics, these substitutions differentially affect the enzyme's selectivity towards lysine and arginine substrates. However, in contrast to predictions, the selectivity is not dominated by the K_m but rather by the k_{cat} values; mutants containing alanine 226 have a dramatically decreased k_{cat} . Additionally, the role of aspartic acid 102 in the catalytic triad of trypsin was tested by replacing it with asparagine. The resulting enzyme has similar K_m values but only five per cent of the k_{cat} value of the parent enzyme in catalysing the hydrolysis of arginine and lysine peptide substrates.

In experiments with rat carboxypeptidase A, the substitution of phenylalanine for tyrosine 248, a residue which has been considered a potential proton donor in the catalytic action of the enzyme for peptide substrates, results in a mutant enzyme that is still competent at hydrolysing a peptide substrate and two ester substrates, although it has an approximately 15-fold reduced activity towards potato carboxypeptidase inhibitor (S. Gardell, D. Hilvert & W.J. Rutter, personal communication). Replacement by alanine of threonine 51 of the tyrosyl-tRNA synthetase of *Bacillus stearothermophilus* results in a lowering of the K_m for the substrate ATP by a factor of two⁶. More dramatically, the K_m for ATP in the amino-acylation step is decreased more than 100-fold by replace-

ment of the threonine by a proline, which probably disrupts an α -helix in the region of the enzyme where residue 51 is located.

The effect on *E. coli* dihydrofolate reductase of substituting lysine for glutamine 139, which participates in a hydrogen-bonded salt bridge with histidine 141 remote from the active site, is a significant decrease in stability for the urea-induced unfolding transition (S.J. Benkovic, C.R. Matthews & D. Tu personal communication). The thermal transition for the mutant is 36°C while that of the parent enzyme is 47°C. Most interestingly, at temperatures both above and below the transition zone the catalytic activity of the mutant enzyme is unchanged from that of the parent. The conversion of proline 39 of dihydrofolate reductase to a cysteine produces a mutated enzyme with normal catalytic activity⁷. According to the X-ray structure of the enzyme, the cysteine would be situated in such a way that it should be possible readily to form a disulphide bridge with cysteine 85. Oxidative formation of this disulphide substantially reduces enzymatic activity, which is ascribed to a concomitant reduction in the dynamic flexibility of the enzyme molecule.

While much attention will continue to

be focused on the use of site-directed mutagenesis to modify enzyme active sites, alteration of structural regions should also be important. For relatively small molecules, such as many peptide hormones, where chemical synthesis is feasible, the systematic replacement of substantial segments outside the active site by non-homologous segments that preserve or accentuate the original secondary-structure characteristics has already produced peptides with high biological activity and stability⁸. A similar approach to the alteration of major regions of secondary structure in enzymes by site-directed mutagenesis could lead to enzymes of increased activity and stability. □

1. Polgar, L. & Bender, M. J. *Amer. chem. Soc.* **88**, 3153 (1966).
2. Neet, K.E. & Koshland, D.E. *Proc. natn. Acad. Sci. U.S.A.* **56**, 1606 (1966).
3. Sigal, I.S., Harwood, B.G. & Arentzen, R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7157 (1982).
4. Dalbodie-McFarland, G., *et al.* *Proc. natn. Acad. Sci. U.S.A.* **79**, 6409 (1982).
5. Sigal, I.S., DeGrado, W.F., Thompson, B.J. & Petteway, S.R.Jr. *J. biol. Chem.* **259**, 5327 (1984).
6. Wilkinson, A.J., Fersht, A.R., Blow, D.M., Carter, C. & Winter, G. *Nature* **307**, 187 (1984).
7. Villafranca, J.E. *et al.* *Science* **222**, 782 (1983).
8. Kaiser, E.T. & Kezdy, F.J. *Science* **223**, 249 (1984).

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Oncogenes

Yeast aids cancer research

from Paul Nurse

MUTANT *ras* genes are strongly implicated in the transformation of certain mammalian cells to cancer cells, which can then form tumours. Thus, point mutations are detected in the *ras* genes of some tumour cells, and the mutant genes are capable of completing the transformation of NIH 3T3 fibroblast cells. But the biochemical functions of the p21 (referring to their size) proteins produced from either normal or mutant *ras* genes are not well understood. The discovery of *ras*-related genes in the yeast, *Saccharomyces cerevisiae*^{1,2}, has opened up new and powerful approaches to this problem which are not possible in mammalian cells. Sophisticated genetic procedures available in yeast, such as tetrad analysis and gene replacement, allow the behaviour of specifically mutated genes to be studied in a variety of genetic backgrounds. Two recent papers^{3,4} clearly demonstrate the power of this type of analysis and have provided important clues about the biochemical function of *ras* genes.

Yeast contains two genes closely related to *ras*, *RAS1* and *RAS2* (not *ras1* and *ras2* simply because of the conventions of *S. cerevisiae* geneticists), which code for proteins of 309 and 322 residues^{1,2}. These are around 90 per cent homologous to the mammalian p21 proteins in their first (amino-terminal) 80–90 amino acids, and about 60 per cent homologous over their

first 172 amino acids but are dissimilar at their carboxy-termini. Disruption of both the *RAS1* and *RAS2* genes is lethal to yeast but disruption of only one of them is not^{5,6}; thus, *RAS* functions are necessary for yeast cell viability but these can be provided by either *RAS1* or *RAS2*.

Wigler and his coworkers³ have now shown that yeast cells are still viable when the yeast *ras*-related genes are replaced by a human *ras* gene, *H-ras*. The human gene is not completely effective in replacing the yeast genes; only about 40 per cent of yeast spores containing *H-ras* germinate and those that do grow slowly. This is probably due to differences in the carboxy-terminal regions of the yeast and human genes since there is much better germination of spores containing a chimaeric gene made up of the first part of the human gene and the second part of the yeast *RAS2* gene, and the resulting cells have a normal generation time. The interchangeability of the yeast and human genes establishes that much of the biochemical function of the yeast *RAS* proteins is shared by the mammalian p21 proteins. Further support for this view comes from Scolnick and coworkers who have found that the amino-terminal region of yeast *RAS1* binds guanine nucleotides and has a GTP hydrolytic activity, like mammalian p21⁷.

In a second paper, Wigler and Ishikawa, with their coworkers in the United States

IMAGE
UNAVAILABLE
FOR
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REASONS

Mounds formed by callianasid ghost shrimps on the floor of the lagoon at Ehwetak Atoll, Marshall Islands. The opening of the mound, which is about 15 cm high, is arrowed. The shrimps sort massive quantities of sediment to extract organic matter, burrowing to depths of 1.5 metres. The redistribution of radio-nuclides resulting from this activity is described on page 674 of this issue. Photograph by T.H. Suchaneh.

and Japan describe their investigation of the biochemical functions of the *RAS* proteins in yeast⁴. They find mutants of a gene called *BCY1* that can still grow even though their *RAS1* and *RAS2* genes are disrupted. *BCY1* is one of three yeast genes whose products are involved in the synthesis of cyclic AMP and the phosphorylation of proteins by a cyclic-AMP dependent protein kinase that is composed of a catalytic sub-unit and a regulatory sub-unit. *BCY1* mutants are deficient in the regulatory sub-unit of the cyclic AMP-dependent protein kinase, *CYR3* in the catalytic sub-unit, and *CYR1* in adenylyl cyclase, the enzyme that synthesizes cyclic AMP in the cell⁵. In the absence of cyclic AMP the regulatory sub-unit binds to and inhibits the catalytic sub-unit of the kinase; the enzyme becomes activated when cyclic AMP binds to the regulatory sub-unit and causes its dissociation from the catalytic sub-unit.

CYR1 mutants resemble strains in which both *RAS1* and *RAS2* have been disrupted in that they are lethal but their lethality can be suppressed by *BCY1* mutations. Other similarities between these strains are that both have low levels of cyclic AMP and both sporulate in relatively nutrient-rich medium⁴. Therefore a lack of *RAS* proteins in the cells has effects that are similar to those caused by a reduction in adenylyl cyclase activity. Because other guanine nucleotide-binding proteins have been found to modulate adenylyl cyclase activity, the authors suggest that this may be one of the biochemical functions of the *RAS* proteins. They support this suggestion by showing that cells that contain disrupted *RAS* genes lack a membrane-localized GTP-dependent activity which would stimulate adenylyl cyclase⁴.

What effects are seen when the *RAS* genes of yeast are mutated in a way that is equivalent to the mutations of human *ras* genes that result in cell transformation? To answer this question, a mutant was constructed in which the glycine at position 19 of *RAS2* was replaced with a valine⁴, equivalent to the change at position 12 of p21 that results in mammalian cell transformation. This *RAS2* mutation affects yeast in a similar way to *BCY1* mutants; neither mutant strain can sporulate and, when nutritionally starved, both have low viability, fail to accumulate storage carbohydrates and have unusually high levels of trehalase activity. The *RAS2* mutant strain has four times the normal content of cyclic AMP and has an increased membrane-localized GTP-dependent activity that stimulates adenylyl cyclase. Therefore, a mutant yeast *RAS* protein, which is analogous to a transforming p21 protein, stimulates adenylyl cyclase, leading to high levels of cyclic AMP and probably to increased cyclic AMP-dependent protein phosphorylation in the cell. This is an intriguing conclusion, given that cyclic AMP has been implicated in growth and cell-cycle control of certain mammalian cell types⁹, and suggests that

cyclic AMP-dependent protein phosphorylation may be involved in the transformation of mammalian cells mediated by mutant *ras* genes.

Cell-cycle control

Will it be possible to use the yeast system to delineate the steps by which the transforming mutants of mammalian *RAS* genes eventually stimulate cells that contain them into uncontrolled entry into the cell cycle? A strong case can be made for this view: altered *RAS* genes do seem to have effects on the yeast cell cycle.

Cells of yeast in which both *RAS* genes have been disrupted accumulate in the G₁ phase of the cell cycle prior to DNA replication; disruption of the *RAS2* gene alone pushes cells out of the cycle and into sporulation even in relatively nutrient-rich medium, which normally favours cell proliferation. When glycine 19 of *RAS2* is replaced by valine, cells in nutrient-poor medium leave the cycle without having built up their carbohydrate reserves, and so lose viability and fail to sporulate.

It seems, therefore, that a decrease in cyclic AMP-dependent protein phosphorylation causes yeast to leave the mitotic cell cycle. What proteins could be the target for this phosphorylation? One obvious candidate is the product of the *CDC28* start gene^{10,11}, which is required in G₁ for commitment to the mitotic cycle; its homologue in fission yeast also regulates the initiation of mitosis. Analysis of the sequences of these two genes suggests that they code for a protein kinase that is phosphorylated^{11,12}. If that phosphorylation is stimulated by cyclic AMP-dependent protein kinase, then a direct link could be established between *RAS* and cell-cycle control in yeast. Interestingly, a role for cyclic AMP in cell-cycle control has been proposed previously from studies on α -factor¹³, a mating pheromone of yeast which causes transient arrest in G₁ prior to conjugation¹⁰. Pheromone addition has been reported to inhibit adenylyl cyclase and this could reduce levels of cyclic AMP in the cell, leading to G₁ arrest¹³. Although this observation is consistent with a role for cyclic AMP in the control, more recent work has failed to confirm that α -factor induces an inhibition of adenylyl cyclase¹⁴.

It may not be possible, however, to study in yeast the whole pathway that links *ras* genes to mammalian cell-cycle control, since the controls in yeast and mammalian cells may be rather different. In yeast, the control is fairly simple¹⁰. Apart from the pheromone effects, cell-cycle control is exerted at the level of cellular growth rate, probably by a process of monitoring protein synthesis^{10,15}. When nutrients are available and cells are growing, they enter the mitotic cycle, whilst cells faced with nutrient deficiency leave the mitotic cycle and enter stationary phase or sporulation. The *RAS* genes may influence this control only indirectly by disturbing cellular growth rate. In mammalian cells there is an extra

layer of controls appropriate to a multicellular organism: cell proliferation must be controlled during embryogenesis, wound healing and tissue renewal for example — such as growth factors, hormones and molecules concerned with cell-surface contact — that are not relevant for yeast. Although *ras*-related proteins may be performing similar immediate biochemical functions in yeast and mammalian cells, their longer-range physiological effects may be quite different. It is possible that the *ras* genes have been recruited into the extra layer of controls appropriate to multicellularity at a point in evolution after yeast and the metazoa diverged. □

1. Defeo-Jones, D., Scolnick, E., Koller, R., & Dhar, R. *Nature* **306**, 707 (1983).
2. Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J. & Wigler, M. *Cell* **36**, 607 (1984).
3. Kataoka, T., et al. *Cell* **40**, 19 (1985).
4. Toda, T. et al. *Cell* **40**, 27 (1985).
5. Tatchell, K., Chaleff, D., Defeo-Jones, D. & Scolnick, E. *Nature* **309**, 523 (1984).
6. Kataoka, T. et al. *Cell* **37**, 437 (1984).
7. Temeles, G., Gibbs, J., D'Alonzo, J., Sigal, I. & Scolnick, E. *Nature* **313**, 700 (1985).
8. Matsumoto, K., Uno, I., Oshima, Y. & Ishikawa, T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2355, (1982).
9. Rozengurt, E. *Adv. Cyclic Nucleotide Res.* **14**, 429 (1981).
10. Pringle, J. & Hartwell, L. *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance* (eds. Strathern, J., Jones, E. & Broach, J.) 97 (Cold Spring Harbour, New York, 1981).
11. Lorincz, A. & Reed, S. *Nature* **307**, 183 (1984).
12. Hindley, J. & Phear, G. *Gene* **31**, 129 (1984).
13. Liao, H. & Thorne, J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1898 (1980).
14. Casperson, G., Walker, N., Basier, A. & Bourne, H. J. *biol. Chem.* **258**, 7911 (1983).
15. Unger, M. & Hartwell, L. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1664 (1976).

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100 years ago

CIVILISATION AND EYESIGHT

THE question of the increasing prevalence of short sight has for a considerable time been the subject of much investigation and speculation in Germany, the results of which have been in many cases to give rise to predictions of rather an alarmist tendency. The numerous statistics from German schools have shown that the proportion of short-sighted boys continually increases from form to form, and from this fact it is very generally argued that the continued use of the eyes for the perception of near objects is the essential if not the only factor in the production of short sight. This view appears, again, to be supported by statistics which allot the largest proportion of short-sighted individuals to those branches of industry or those pursuits which constantly call for near vision. Two points, however, appear to be forgotten, or at all events fail to receive sufficient consideration, in arriving at such a conclusion. In the first place, there is an undoubted tendency to increase in the degree of short sight with age alone up to the period of cessation of growth. The second point is how far the greater proportion of short sight amongst literary men, or artisans whose daily work necessitates close vision, is actually due to their occupation, or depends on the circumstances that, being originally short-sighted, they have drifted into pursuits which are more attractive to them, owing to their not being able to enjoy out-door work or sports to the same extent as others whose eyes are more fortunately focussed. From *Nature* **31** 387, 26 February 1885.

Nuclear physics

Spinning the erbium nucleus into different shapes

from Neil Rowley

IT HAS long been known that the motions of individual neutrons and protons within the nucleus are strongly affected when the nuclear matter distribution is bodily rotated. At high enough rotational velocities, however, the entire matter distribution itself may undergo convulsive changes, as demonstrated by a recent experiment (Simpson, J., *et al. Phys. Rev. Lett.* 53, 648; 1984), performed at the Nuclear Structure Facility of the Science and Engineering Research Council by a group from the Universities of Liverpool and Copenhagen.

The predicted evolution of the shape of a typical deformed rare-earth nucleus with increasing rotational frequency ω is shown in Fig.1. The nucleus is shown rotating in the plane of the page so that we are looking along the axis of rotation. At low frequencies (Fig.1a) the matter distribution has a 'prolate' shape, that is it has an axis of symmetry s with its poles stretched out along this axis. At intermediate spins (Fig.1b) the rotation forces some of the matter out into the plane of the page. The original axis s is still readily recognizable but is no longer an axis of rotational symmetry; the nucleus is now said to be 'triaxial'. At high spins (Fig.1c) the matter distribution is more extensively thrown out in the plane of the page but in a symmetrical fashion, so that no special direction in the plane now exists. The nucleus again possess a symmetry axis but this now points out of the plane so that it coincides with the axis of rotation and the system is flattened at its poles into a 'oblate' shape. It is the dramatic collapse of the erbium (^{158}Er) nucleus into such an oblate configuration that Simpson *et al.* have observed.

In order to understand the experimental signature for such a shape change, one must consider the concept of 'rotational frequency' for a microscopic object. The excited states of the nucleus should strictly be labelled by their total angular momentum I rather than by ω , which is a classical quantity. If, however, one does picture the nucleus as a rotating deformed object, then its positive charge will result in the generation of a rotating deformed electric field which will emit electromagnetic radiation. The rotational frequency of such a deformed object may be inferred from the frequencies of the sequence of γ rays that the nucleus emits as it decays from states at high angular momentum.

If the rotational frequency is a meaningful concept, the properties of the nucleus should vary in a smooth manner as a function of it. Fig.2 shows the total energy of

^{158}Er relative to that of a classical rotor with the same spin and frequency. Despite the two rapid increases displayed, we see that this quantity is indeed a smooth continuous function of ω . Such rapid changes have been known for some time (both in this nucleus and in other systems) and are qualitatively well understood. They correspond to a pair of neutrons (at ω_1) and a pair of protons (at ω_2) starting to rotate around the axis of collective rotation and thus contributing a large fraction of the total angular momentum of the system. This process, known as rotational alignment, tends to localize these nucleons in the

plane of rotation and thus contributes to the triaxiality of the system (see Fig.1b).

The highest point marked in Fig.2 corresponds to a nuclear spin of $38\hbar$. Although the Liverpool-Copenhagen experiment has quite clearly shown several excited states at higher spins (above ω_{po}), they are excluded from Fig.2 because the smooth nature of the curve completely breaks down above this point. It is precisely the observation of this breakdown that indicates that the nucleus has flipped over into an oblate shape. This can be understood from Fig.1c. Since the matter distribution is symmetrical about the axis of rotation, then so is the associated electric field. Consequently, the rotation does not change this field and cannot produce radiation; thus, γ decays from a nucleus in such a configuration must come from changes in the orbits of individual nucleons moving within the matter distribution rather than from a collective rotation of this distribution itself. There is no reason, therefore, why the fre-

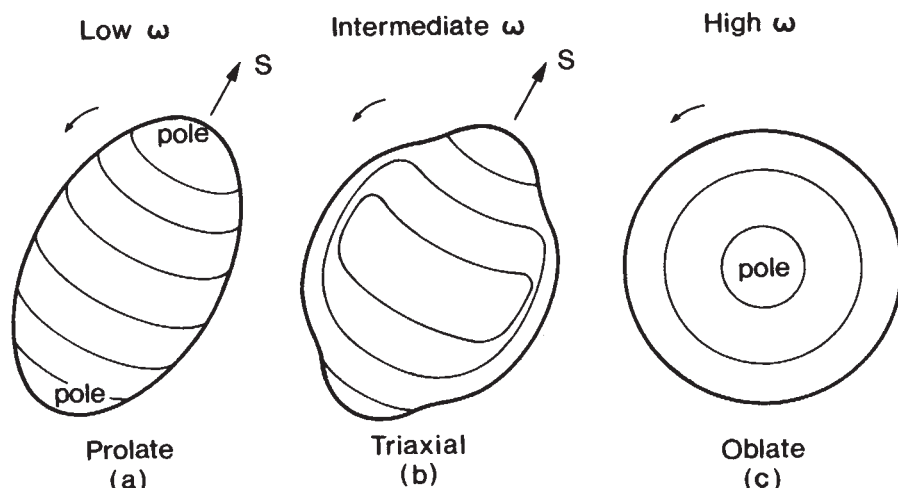
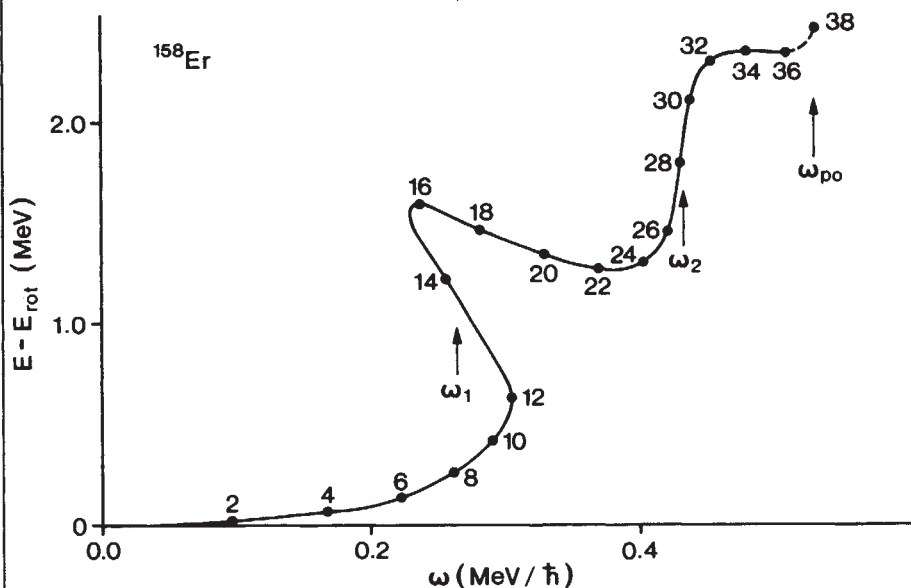


Fig.1 Change in shape of a typical rare-earth nucleus as the nuclear rotational frequency ω , increases. Explanations in text.

Fig.2 Energy of the ^{158}Er nucleus, E , relative to that of a classical rotor E_{rot} with the same spin, I and frequency, ω . Spin values are marked along the curve in units of $\hbar$.



quencies of the γ rays should be characterized by a 'collective rotational frequency', and a more erratic behaviour ensues.

Indeed, the motion of individual nucleons is principally determined by the average field of all the others, and for rotations about a symmetry axis this field is also unchanged. Thus, in a sense, the nucleons are completely unaware of the rotation and, to some extent, it is meaningless even to speak of the system rotating. It is preferable to say that the total angular momentum of the nucleus is aligned along the symmetry axis.

At much higher angular momenta, fission of the nucleus must eventually occur. Breaking of the nucleus into two distinct fragments clearly corresponds to a prolate configuration. Prior to the fission, therefore, one can expect the matter distribution to reassume a collectively rotating prolate

shape (as in Fig. 1a) with a much increased deformation. Strong evidence for such a 'superdeformed' state in ^{152}Dy has been found in an earlier experiment performed at the Nuclear Structure Facility (Nyako, B.M. *et al. Phys. Rev. Lett.* **52**, 507; 1984).

Both of these experiments owe their success to a unique γ -ray spectrometer, TESSA2 (Twin, P.J. *et al. Nucl. Phys. A* **409**, 343; 1983). This was designed by members of Liverpool University to suppress much of the large γ -ray background produced by Compton scattering in the detectors. The enormous power of such a device has been recognized by many other groups and similar devices are currently under construction in Europe, North America and Scandinavia. $\square$

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Astrophysics

Unsmoothing the Universe

from Virginia Trimble

THE world around us is highly structured on many different scales. On some scales, we think we understand, or at least can reduce, the problem to basic physics: atoms and molecules follow from quantum electrodynamics, the masses of stars are determined by a balance between thermodynamic pressure and gravity. But the origin of the largest structures — galaxies and clusters of galaxies — remains a mystery, and a basic one, for they may be the oldest density fluctuation of all, without which the kinds of stars and planets we now see would have little chance of forming.

Several speakers at the most recent Texas Symposium on Relativistic Astrophysics* suggested ways galaxies might have formed. All sounded about equally plausible, and all required at least two somewhat special circumstances ("tooth-fairies" according to David Schramm, University of Chicago) to yield the observed degree of lumpiness on the right scales at the right time. This suggests that we may still lack the right underlying physics. There were also disputes on precisely what data need to be explained.

The points on which nearly everyone agrees are, first, that galaxies must form before a redshift of 3.5–4 in order for us to see quasars at that epoch. Second, if the universe has passed through an inflationary phase, the luminous matter must be more highly clustered than the dark matter making up most of the critical density. Third, the amplitude of the density fluctuations, $\delta\rho/\rho$, must by now have grown to unity, or larger, on the cluster and supercluster scales. And fourth, the amplitude of fluctuations (at least adiabatic ones) must have been less than a few parts in a thousand at

the time matter and radiation decoupled (redshift ~ 1000) in order not to produce anisotropies in the microwave background radiation larger than the current upper limits, $dT/T = 2 \times 10^{-5}$ over angular scales 1–4 arc min, according to a review by Bruce Partridge (Haverford College).

Some of the possible models and difficulties with them include:

(1) Strings are hypothetical thin tubes of false vacuum left over after a universal phase change to a real vacuum. They connect pairs of monopoles and bound domain walls. They can cross over themselves and reconnect, breaking off loops. Alexander Vilenkin (Tufts University; see also *Nature* **312**, 598; 1984) discussed how oscillating closed loops can act as condensation centres for galaxy formation. A virtue of the idea is that the reconnection process guarantees that lumps formed around loops will have non-random phasing; that is, that galaxies will be more highly clustered than matter as a whole. But the strings themselves contribute nothing to the local mass-density, so that we must still postulate some form of dark, non-baryonic matter that makes up most of the universe.

(2) 'Bias' is the idea that galaxies might form only at extreme points of the density distribution. This yields galaxies that are more clustered than the mass in general, even with a randomly-phased spectrum of primordial perturbations, because galaxy-sized lumps superimposed on larger-scale positive density fluctuations have the best chance of collapsing. An inflationary epoch automatically expands quantum fluctuations to give the requisite initial spectrum (equal power at each scale as it comes within the horizon) and though we cannot currently calculate the amplitude of the fluctuations, the microwave isotropy sets

an upper limit. But the need for some kind of weakly-interacting massive particle to make up most of the energy density constitutes a second tooth-fairy.

James Bardeen (University of Washington, Seattle) and Simon White (University of Arizona) presented biased models of this type. In Bardeen's model, with cold dark matter — axions, super-symmetric particles (see *Nature* **313**, 9; 1985), or other particles of ≥ 2 GeV — either isocurvature or adiabatic fluctuations, together with gravitational interactions, suffice to form galaxies with the observed distribution in the sky. But structures as large as Abell clusters could only have collapsed by the present time if they had much larger density contrasts over the background than observed. White's cold models behave similarly, while those dominated by particles of low mass (for example, neutrinos ≤ 100 eV) or intermediate mass (for example, gravitinos ~ 100 MeV) concentrated much of the matter into 10^{16} – 10^{17} Solar mass structures (larger than we see), and produce excessively high density contrasts on smaller scales, unless galaxy formation is strongly suppressed in high density regions — a sort of inverse bias.

(3) Other possible combinations were addressed by Keith Olive (Fermilab) and David Schramm: a universe dominated by cold massive particles that decay to lower-mass relativistic particles just after galaxy formation ends (so that density contrast stops growing), or a universe dominated by low-mass hot particles, but which only starts with small-scale isothermal perturbations. The former must be very finely tuned whereas the latter cannot be produced by inflation or any other known physical process, so again we need two tooth-fairies.

Perhaps the greatest surprise to most participants was a suggestion from Margaret Geller (Center for Astrophysics) that the supposed data these simulations are designed to match are not as secure as we had thought. She has re-examined details of the Shane-Wirtanen galaxy counts, a major source of published statistics which shows how the distribution of galaxies in the sky differs from randomness. It is based on plates that were taken under a wide range of observing conditions and counted by two different people over some period of time. Shane and Wirtanen attempted to compensate for the inevitable non-uniformities by forcing the closest possible matches in regions of plate overlap. One result is to push down the numbers in plates surrounding a bad one.

Geller finds that these and other problems in the original data not only create artificial features in the galaxy distribution but also hide real ones. For instance, a conspicuous filamentary feature traces down the edges of a group of plates counted by Wirtanen rather than Shane; a bad plate near the Coma cluster has suppressed nearby areas and makes the cluster look very isolated; and there is a shift between counts done on single plates and those done across plate

*The Twelfth Texas Symposium on Relativistic Astrophysics, 17–21 December 1984, Hebrew University, Jerusalem.

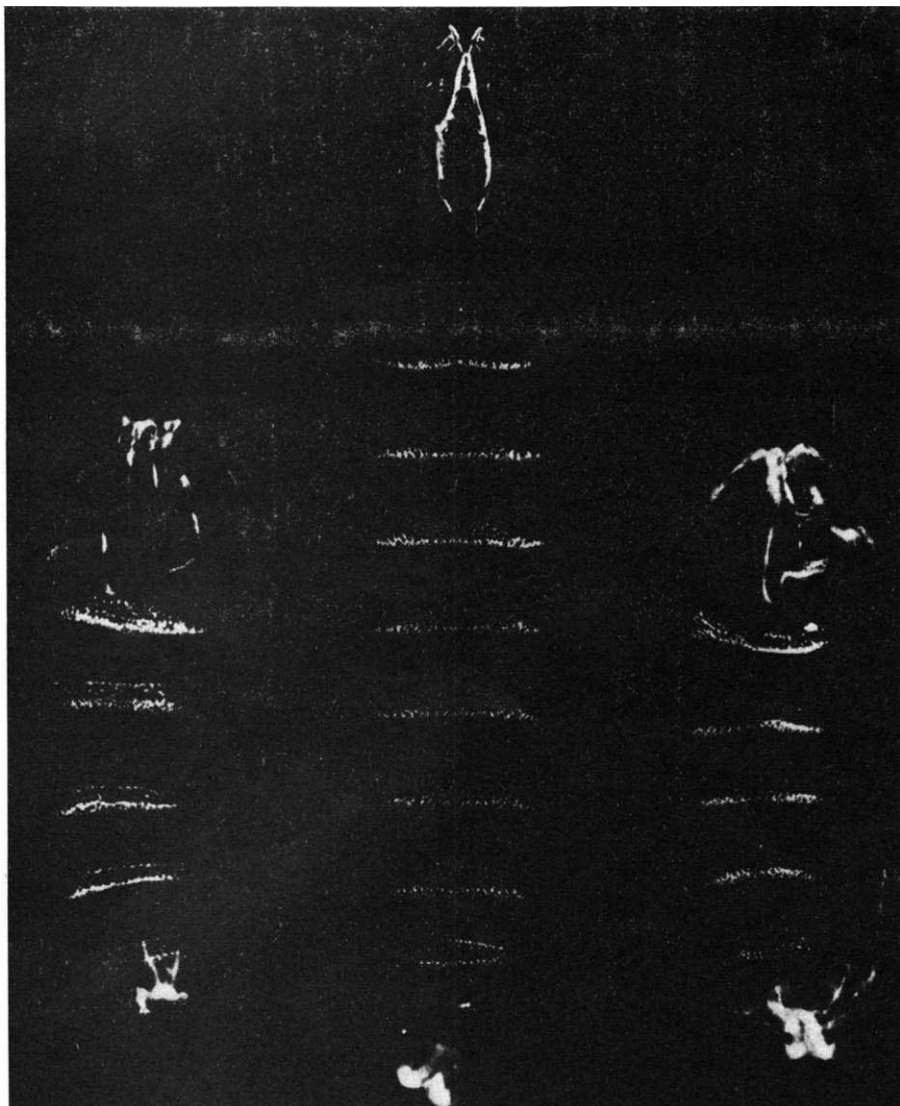
boundaries at just about the scale where one might have expected to see a real change in the slope of the correlation function. Thus only effects that have been seen in more than one survey should be taken seriously, such as the slope of the correlation function ($r^{-1.8}$), but not its precise amplitude, the length scale at which it turns over (if it does), or whether it goes negative at some large distance.

Clusters of galaxies are, themselves, even more strongly clustered than the galaxies, according to Neta Bahcall (STScI). Rich clusters are more clumped than poorer ones. That is, the amplitude of the rich-cluster correlation function exceeds that of the galaxy correlation function by a factor of about 30, at a scale length of 5Mpc; the amplitude for smaller clusters falls in between; and the slope of all three correlation functions is $r^{-1.8}$. Bahcall interprets this to mean that a large fraction of galaxies form outside clusters. But Schramm pointed out that, when the correlation functions are renormalized to scale length as a fraction of the average separation of the objects concerned (a technique adopted from condensed-matter physics), rich and poor clusters look the same, while the galaxies are more strongly clustered, reflecting the action of an extra factor, presumably gravity. If this is correct, then the galaxy correlation function should go negative at about 40 Mpc and that of the cluster at 400 Mpc. As Geller noted, some surveys show such a turn-over but others do not.

Finally, Bahcall remarked that clustering on still larger scales should reveal itself through large velocity deviations from smooth expansion. A hint of the predicted peculiar velocities has recently turned up at another meeting[†]. Marc Aaronson (University of Arizona) and Jeremy Mould (California Institute of Technology) have analysed redshift data for 10 nearby clusters, the distances of which have been determined from the correlation of infrared luminosity with 21 cm line width (the Tully-Fisher relation). Scatter in the derived value of the Hubble constant is minimized if the Local Group of galaxies has a velocity of 788 ± 188 km/s in the direction of galactic latitude $18^\circ \pm 13^\circ$ and longitude $255^\circ \pm 17^\circ$. This is in reasonable agreement with the 600 ± 30 km/s in about the same direction that seems to be our velocity relative to the 3 K microwave radiation. But, more to the point, it can be broken up into a vector sum of an infall of the Local Group toward the centre of our (Virgo) supercluster at about 300 km/s plus a somewhat larger motion of the supercluster towards the adjacent Hyades-Centaurus supercluster. The best-fit value of the Hubble constant from this analysis is about 90 km/s/Mpc, which presents its own problems. But that's another story. □

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[†]The 165th meeting of the American Astronomical Society, 14-16 January 1985, Tucson, Arizona.



Mutants without mutations

THE *Drosophila* embryo shown on the left is an example of a *Krüppel* mutant, the result of a mutation in the *Krüppel* gene. This embryo is short relative to the normal embryo shown in the middle, because it lacks all thoracic and several abdominal segments, so that the head is directly connected to one of the more posterior abdominal segments. The embryo on the right, which resembles the *Krüppel* mutant, in fact has normal *Krüppel* genes, but is the dramatic result of the injection of the embryo, at a very early stage in its development, with a large dose of what is known as *Krüppel* 'antisense' RNA, as described on page 703 of this issue.

Krüppel 'antisense' RNA, which is synthesized *in vitro* from the 'wrong' strand of *Krüppel* cDNA, is complementary in sequence to *Krüppel* 'sense' (natural) RNA and so is able to pair with it to form double-stranded RNA. Although the precise fate of *Krüppel* antisense RNA in injected embryos is unknown, it is thought to pair with *Krüppel* sense RNA and so prevent it from being translated into the normal *Krüppel* gene product. Direct evidence that antisense RNA (for beta globin) works in this way in microinjected frog oocytes has recently

been reported by D.A. Melton (*Proc. natn. Acad. Sci.* **82**, 144; 1985).

The ability to 'phenocopy' mutants in this way is of more than academic interest. An example of the kind of problem to which the technique might be applied comes from some recent work on the *engrailed* locus of *Drosophila*, which was discussed by Peter Lawrence in these pages three weeks ago (24 January, p.268). *Engrailed* has some characteristics of a homoeotic gene, and, like the better known homoeotic genes of the bithorax and Antennapedia complexes, it contains a homoeo-box sequence. Close by the *engrailed* gene there is a second homoeo-box sequence, which identifies the *engrailed-related* gene. What does this gene do? Nobody knows, for as yet no mutations mapping in this region of the *Drosophila* genome have been identified. The use of antisense RNA offers a direct way to test what effect the loss of *engrailed-related* gene function has on development. Antisense RNA should also be useful in dissecting the activities of genes involved in the regulation of development by preventing their expression at specific times and in discrete regions of the embryo.

Geoffrey North

Molecular turnover and memory

SIR — Francis Crick considers in *News and Views* under the title "Memory and molecular turnover"¹ the problem, often overlooked, that although memory operates over periods of years or decades, most macromolecules (with the exception of DNA) turn over with half lives of hours or weeks. Crick sees the dilemma since memory is prolonged and a consequence of inter-synaptic interaction which is dependent on fixed intrasynaptic macromolecules, such as membrane glycoproteins, either singly or more probably in larger aggregates of some form. To sustain memory two alternative strategies are proposed, either the memory macromolecule is immune from turnover (a less likely possibility for Crick) or the memory macromolecules in a synapse can be replaced one at a time without altering the overall state of the memory macromolecular complex.

My response to Crick's interesting challenge lies in the observation that perinuclear (often sided) membrane disposition is required *before* protein catabolism in normal cells takes place² and some special association of proteins with cytoskeletal elements may precede routing to the cellular destructive machinery^{3,4}.

Nerve cells are exquisitely polarized with the cell body (nucleus, polysomes and Golgi) quite spatially distinct from synapse forming processes such as axons or dendrites. Therefore, memory proteins dispatched from around the nucleus into processes by axoplasmic flow would need to retrace their steps for destruction as described in the protein turnover cycle⁵. It is not at all fanciful to suppose that the intricate arborizations of nerve cell processes in the temporal cortex (and everywhere else) have evolved, at least in part, in order to separate and 'immunize' informational macromolecules spatially from the apparatus of molecular turnover. Simply detaching such macromolecules from the neuroskeletal system would suffice (compare refs 2-4), thereby preventing (or slowing) the return of the macromolecules to the perinuclear destructive machinery. Alternatively, selective reversible detachment-attachment to the neuroskeleton would identify populations of proteins (or individual proteins) which are to be routed for destruction and replacement.

Both of the above alternatives could be mediated by protein modifications such as those described by Crick. Likewise both possibilities could operate together in a single neurone. Either way, neuronal evolution could have capitalized on the spatial separation of proteins (in membranes) in the synapses of cellular processes away from the perinuclear destruction apparatus in cell bodies so that information storage is achieved by permanent or temporary macromolecular stabilization.

It is perhaps salutary in an ageing human

population that facultative loss, as occurs in Alzheimer's disease, and motor disorder, as seen in Parkinson's disease, might be triggered by the disruption of the status of cell body-synaptic trafficking in cortical neurones and nigro-striated pathways respectively, leading to intraneuronal degradative (degenerative) processes.

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1. Crick, F. *Nature* **312**, 101 (1984).
2. Evans, P.J. & Mayer, R.J. *Biochem. J.* **216**, 151-161 (1983).
3. Rogers, S.W. & Rechsteiner, M. in *Intracellular Protein Catabolism* (eds Bond, J.S., Bird, J.W.C. & Khairallah, E.) (Liss, New York, in the press).
4. Doherty, F. & Mayer, R.J. *Biochem. J.* (in the press).
5. Mayer, R.J., Evans, P., Russell, S. & Amenta, J.S. *Ciba Fdn. Symp.* **103**, 202-219 (1984).

Human B-cell cytotoxic lymphokine priority

SIR — The recent article on the cloning and expression of human lymphotoxin by scientists from Genentech Inc.¹ described work that first came to my attention when a report appeared in *The Guardian* of 6 June 1984 concerning an announcement by the company that they had developed a new cancer drug which did not have side effects. The initial scientific papers from Genentech² stated that the lymphotoxin in question had a relative molecular mass (M_r) of approximately 20,000. I therefore thought that it must be a new cytotoxic lymphokine. Some seven years earlier^{3,4}, I had published a description of a humoral cytotoxic factor produced by a human lymphoblastoid cell line of B-cell lineage derived from a local patient with leukaemia.

Those papers of mine, which characterized the properties of the factor and how it was distinguished from other forms of cell killing, were probably the first well documented studies showing that some human B-lymphoblasts growing *in vitro* produced a cytotoxic lymphokine. In these early papers we also reported that the humoral factor preferentially kills malignant cells and that it had reduced by approximately 50 per cent the incidence of malignancy (fibrosarcoma) in mice^{3,4}. Our studies also showed that the cytotoxic factor was a protein with an M_r of $65,000 \pm 1,000^5$.

In the recent paper in *Nature* by Genentech, the authors state that their initial published result (1984) on the relative molecular mass was incorrect and that the actual M_r of this human lymphoblastoid cell-derived lymphotoxin is 60,000-70,000. This value is so closely similar to the 65,000 M_r we had previously reported in 1980⁵, that it now seems extremely likely that the human B-lymphoblast-produced lympho-

toxin — cloned recently by Genentech — is identical with the cytotoxic factor first described in 1977^{3,4}. It will be for future studies to establish whether the cytotoxic factor described from Cambridge in 1977 and the lymphotoxin described in 1984 in the United States are in fact identical and to explore the efficacy in the treatment of malignant states.

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1. Gray, P.W. *et al.* *Nature* **312**, 721 (1984).
2. Aggarwal, B. B. *et al.* *J. biol. Chem.* **259**, 686 (1984).
3. Karpas, A. *Brit. J. Cancer* **35**, 152 (1977).
4. Karpas, A. *Brit. J. Cancer* **36**, 437 (1977).
5. Neumann, H. & Karpas, A. *Bioch. J.* **194**, 847 (1981).

HTLV-III, LAV, ARV are variants of same AIDS virus

SIR — Retroviruses have been isolated reproducibly from patients with the acquired immunodeficiency syndrome (AIDS), and have been designated human T-cell lymphotropic virus (HTLV) type III¹, lymphadenopathy-associated virus (LAV)², or rather recently AIDS-related virus (ARV)³ by different groups of investigators. Forty-eight independent HTLV-III isolates were originally reported from our laboratory¹, several additional ones since^{4,5}, and now we have obtained more than 100 independent isolates (S.Z. Sakhuddin *et al.*, in preparation). The recent publications of the complete nucleotide sequence of two HTLV-III proviruses⁶, LAV⁷, and ARV⁸ allows a detailed comparison (see table). Sequences of HTLV-III clones BH5 and BH8 (representing the 5' and 3' portions of provirus(es), respectively), clone LAV1a, and ARV-2 are compared to HTLV-III clone BH10. LAV is closely related to HTLV-III clone BH10 and differs in 1.5% of nucleotides and 2.2% of amino acids, while ARV-2 differs in 6.3% of its base pairs and 9.2% of its amino acids from that of HTLV-III clone BH10. These data show that HTLV-III, LAV, and ARV are variants of the same virus. The greater sequence divergence of ARV from HTLV-III is not likely to be a result of errors in sequence determination. First, sequences obtained independently in different laboratories for the same HTLV-III clones were in agreement⁶. Second, multiple clones of ARV isolated from the same cell line infected with a virus isolate from a single individual differ in sequence from one another by only 2 or 3 base pairs (bp)⁸. Third, we have sequenced another proviral clone of HTLV-III derived from another one (RF) of the original 48 isolates reported¹, which differs from BH10 to a similar degree as does ARV (our unpublished observations with B. Starcich, B. Hahn

AIDS virus sequences

	No. and % (in parentheses) of differences compared with HTLV-III clone BH10 sequence										
	HTLV-III clones BH5/BH8			LAV			ARV				
	Total nucleotides	Total amino acids	Nucleotide differences	Amino acid differences	Non-conservative amino acid differences*	Nucleotide differences	Amino acid differences	Non-conservative amino acid differences*	Nucleotide differences	Amino acid differences	Non-conservative amino acid differences*
LTR†	634	—	8† (1.6)	—	—	10 (1.6)	—	—	30 (4.7)	—	—
Leader and tRNA PBS†	152	—	4† (3.6)	—	—	5 (3.3)	—	—	14 (9.2)	—	—
<i>gag</i>	1,536	512	19 (1.2)	7 (1.4)	3 (0.6)	46‡ (3.0)	16‡ (3.1)	2 (0.4)	86‡ (5.6)	32‡ (6.3)	8 (1.6)
<i>pol</i>	3,045	1,015	29 (0.9)	12 (1.1)	3 (0.3)	59‡ (1.9)	21‡ (2.1)	5 (0.5)	134‡ (4.4)	51‡ (5.0)	14 (1.4)
<i>sor</i>	609	203	6 (1.0)	4 (2.0)	1 (0.5)	2 (3.3)	0 (0.0)	0 (0.0)	31 (5.1)	20 (9.8)	8 (3.9)
Between <i>sor</i> and <i>env-lor</i>	584	—	16 (2.7)	—	—	11 (1.9)	—	—	49 (8.4)	—	—
<i>env-lor</i> :											
Signal peptide	111	37	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.8)	2 (5.4)	0 (0.0)	26§ (23.4)	12§ (32.4)	3 (8.1)
Extracellular portion	1,443	481	27 (1.9)	12 (2.5)	5 (1.0)	32¶ (2.2)	14¶ (2.9)	6 (1.2)	164# (11.4)	82# (17.0)	41 (8.5)
Transmembrane portion	1,035	345	9 (0.9)	6 (1.7)	2 (0.6)	9 (0.9)	5 (1.4)	2 (0.6)	66 (6.3)	42 (12.2)	19 (5.5)
3' <i>orf</i> **	648	216	12 (1.8)	5 (2.3)	1 (0.5)	13 (2.0)	8 (3.7)	3 (1.4)	52†† (8.0)	29†† (13.4)	10 (4.6)
Total	9,213	2,593	122 (1.3)	40 (1.5)	14	144 (1.5)	58 (2.2)	15	582 (6.3)	239 (9.2)	93

* Considered conservative substitution if same charge or both neutral, and both either hydrophilic or hydrophobic amino-acid insertions and deletions not counted.

† Portion of R, U5 and leader sequence deleted from unintegrated HTLV-III clones BH5, BH8 and BH10, and determined from integrated clone HXB2.

‡ Deletion of one copy of 36-bp (12 amino acid) direct repeat sequence.

§ Insertion of 9-bp sequence and deletion of 15-bp sequence.

|| Deletion of 15-bp (5 amino acid) sequence.

¶ Insertion of 15-bp (5 amino acid) sequence.

Deletion of 18-bp (6 amino acid) sequence, insertion of 12 bp (4 amino acid) sequence, deletion of 6 bp (2 amino acid) and 15 bp (5 amino acid) sequences.

** Includes full open reading frame defined by BH8 and LAV sequences; BH10 has a termination codon at amino acid position 134.

†† Insertion of 12-bp (4 amino acid) sequence.

and G. Shaw).

The DNA sequence data confirm the findings from restriction enzyme site analysis of the HTLV-III genomes which show a spectrum of diversity from closely related to more distantly related variants (ref. 9 and F.W.-S. *et al.*, unpublished). The closer similarity of the LAV DNA sequence to that of HTLV-III might be because the individuals from whom these isolates were derived acquired the virus at a similar time and place. In fact, many of our earliest HTLV-III isolates were all from specimens obtained in late 1982 or early 1983 from the east coast of the United States^{1,10} and LAV, although isolated from a French man with a lymphadenopathy syndrome, had his contact in New York in the same period². In contrast, the individual from whom ARV was derived was from California, and the specimen was apparently obtained in 1984³. The other more divergent virus (RF) was obtained from a Haitian patient in 1983.

A comparison of the ARV sequence with that of HTLV-III reveals the greatest nucleotide sequence conservation in the LTR and the *gag*, *pol*, and short open reading frame (*sor*) genes. Many of the differences between the AIDS virus sequences represent in-frame deletions and insertions (see notes at bottom of table). The non-coding areas are somewhat less conserved. The most divergence, however, is within the open reading frame,

designated *env-lor*, which encodes the precursor to the envelope proteins and possibly a second protein analogous to *lor* in HTLV-I, HTLV-II, and bovine leukaemia virus (BLV). Of note is the high level of heterogeneity in the extracellular portion of the envelope which differs in 17.0% of its predicted amino acids (8.5% non-conservative amino acid differences) from that predicted for HTLV-III clone BH10. These data may have significant implications for immune system interactions with AIDS virus infected cells, as well as in the design of reagents for viral detection and treatment.

The sequence of clone H9pv22 also derived from HTLV-III-infected H9 cells shows 0.5% nucleotide and 0.6% amino-acid differences from that of clone BH10 (ref.11).

The presence of a *trans*-acting and transcriptional enhancer in HTLV-III-infected cells, as was demonstrated in HTLV-I, HTLV-II and BLV-infected cells, suggests that there is a gene for *lor* in the HTLV-III genome that may mediate this activity¹². We have hypothesized that the 3' portion of the open reading frame designated *env-lor* may be responsible for this function⁶, though others have not made a similar interpretation^{7,8,11}.

The similarities and differences of the AIDS virus isolates to other retroviruses, including the HTLV-BLV family, are discussed in the recent publications⁶⁻⁸. The

important general structural features of this virus, most notably juxtaposition of myrgagtin, p17, and largagtin, p24 (see ref. 13 for terminology) and the presence of a *lor* coding sequence, suggest that these viruses are related to other members of the HTLV-BLV family. In addition, we are confident that HTLV-III shares nucleotide sequence homology with the *maedi-visna* lenti-retrovirus¹⁴, although another report shows no homology of LAV to *visna*¹⁵. We cannot explain this discrepancy. A more comprehensive discussion of factors to be considered in taxonomic assignments for this virus is forthcoming (W.A. Haseltine *et al.*, in preparation).

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1. Gallo, R.C. *et al. Science* **224**, 497 (1984).
2. Barre-Sinoussi, F. *et al. Science* **220**, 868 (1983).
3. Levy, J.A. *et al. Science* **225**, 840 (1984).
4. Groopman, J.E. *et al. Science* **311**, 447 (1984).
5. Redfield, R.R. *et al. J. Am. med. Ass.* (in the press).
6. Ratner, L. *et al. Nature* **313**, 277 (1985).
7. Wain-Hobson, S. *et al. Cell* **40**, 9 (1985).
8. Sanches-Pescador, R. *et al. Science* **227**, 484 (1985).
9. Shaw, G.M. *et al. Science* **226**, 1165 (1984).
10. Popovic, M. *et al. Science* **226**, 494 (1984).
11. Myesing, M.A. *et al. Nature* **313**, 430 (1985).
12. Sodroski, J. *et al. Science* **227**, 171 (1985).
13. Oroszolan, S. *et al. in Retroviruses in Human Lymphoma/Leukemia* (ed. Miwa, M.) (Japan Scientific Society Press, Tokyo, in the press).
14. Gonda, M.A. *et al. Science* **227**, 173 (1985).
15. Alizon, M. *et al. Nature* **312**, 757 (1984).

The mitochondria and cellular calcium

SIR — Somlyo's *News and Views* article¹ summarized evidence that the regulation of intracellular free calcium level by the sarcoplasmic reticulum in muscle may be carried out by the endoplasmic reticulum in non-muscle cells. I believe the subsidiary role accorded to the mitochondria in this presentation fails to give due weight to recent advances in the study of their Ca-transport. Newer work shows two important features. First, in contrast to the earlier results with the isolated organelles, both Ca affinity and uptake rate are sufficient to predict mitochondrial involvement in intact cell Ca regulation. Second, such regulation can be demonstrated in suspensions of mildly disrupted cells leaving the mitochondria *in situ* with activity observed under very near physiological conditions (see refs 2,3 for reviews). These comments do not detract from the role for the endoplasmic reticulum and sarcoplasmic reticulum in cellular Ca homeostasis summarized by Somlyo, but allow for interactions between organelles particularly in dynamic aspects of Ca-regulation.

One paper specifically cited in Somlyo's article (ref. 4) as negative evidence for a mitochondrial involvement in Ca-regulation merits discussion since much depends upon the precise detail. The Ca-content of mitochondria in resting and activated smooth muscle was determined by electron probe microanalysis. It rose from 0.8 ± 0.5 mmol per kg dry wt to 1.8 ± 0.6 ($n=61$; $\pm$ s.e.m.) which was quite reasonably stated to be "not significant" (in the statistical sense; the *P* value only approaches the 0.1 level). (Somlyo and his group have made exhaustive studies⁵ of the limitations of this technique.) In fact the wrong null hypothesis has been applied: since the authors wish to show that "Mitochondria do not accumulate significant Ca concentrations in normal cells" (the article's title), the data should be assessed for similarity rather than difference. On this basis the conclusion represented by the title would have to be rejected, even if the converse remains unproven. (Recently published revised data⁶ do not materially affect these comments.) A change of the order of 1mM in mitochondrial Ca would not be resolved reliably by this technique but would actually be sufficient to account for much of the Ca necessary to produce full relaxation of cardiac (and possibly smooth) muscle given the relative volumes occupied by the mitochondria in these tissues and the concentration of binding sites saturated during contraction⁷. Other negative evidence, to which Somlyo attaches significance, includes reports that Ca-uptake rates and affinities are too low, particularly at physiological $[Mg^{2+}]$ ⁸. This evidence has been superceded; even the symposium papers⁹⁻¹¹ immediately preceding Somlyo's own contribution⁴ provide strong, positive evidence for the physio-

logical role of the mitochondria. In particular, Becker¹¹ demonstrates $[Ca^{2+}]$ regulation at submicromolar levels by both isolated mitochondria and those accessed by selective (digitonin) perforation of the cell membrane under near physiological conditions.

These phenomena have recently been studied with selectively 'skinned' cardiac muscle either as myocyte suspensions (ion fluxes monitored with ion-sensitive electrodes¹²) or in trabeculae (force measurements^{13,14}) under physiological conditions of $[Mg^{2+}]$, $[Na^+]$, pH, temperature and extramitochondrial Ca-buffer capacity. 'Sarcoplasmic' $[Ca^{2+}]$ reduction in the micromolar range by the mitochondria has an effective time constant below 200 ms. Transfer of Ca from the mitochondria to the sarcoplasmic reticulum, as a result of their different Ca-affinities and $[Na^+]$ sensitivity, can occur between successive calcium releases from the reticulum. Thus Ca is only transiently accumulated by the mitochondria during relaxation. I believe that the evidence reviewed should keep minds open to the idea that the "complicated mitochondrial Ca transport system so passionately pursued by biochemists for more than 20 years" (ref.1, p.517) has physiological significance, and may be an integral part of the system responsible for cellular Ca regulation.

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1. Somlyo, A.P. *Nature* **309**, 516-517 (1984).
2. Nicholls, D. & Akerman, K. *Biochim. biophys. Acta* **683**, 57-68 (1982).
3. Carafoli, E. in *Membrane Transport of Calcium* (ed. Carafoli, E.) 109-139 (Academic, London, 1982).
4. Somlyo, A.P., Somlyo, A.V., Shuman, H. & Scarpa, A. in *Calcium and Phosphate Transport across Biomembranes* (eds Bronner, F.B. & Peterlik, M.) 87-93 (Academic, New York, 1981).
5. Somlyo, A.P., Somlyo, A.V. & Shuman, H. *J. Cell Biol.* **81**, 316-335 (1979).
6. Bond, M., Schuman, H., Somlyo, A.P. & Somlyo, A.V. *J. Physiol., Lond.* **357**, 185-201 (1984).
7. Fabiato, A. *Am. J. Physiol.* **245**, C1-C14 (1983).
8. Scarpa, A. & Graziotti, P. *J. gen. Physiol.* **62**, 756-772 (1973).
9. Nicholls, D. & Akerman, K. in *Calcium and Phosphate Transport across Biomembranes* (eds Bronner, F. & Peterlik, M.) 83-86 (1981).
10. Becker, G.L. in *Membrane Transport of Calcium* (ed. Carafoli, E.) 79-82 (1982).
11. Lehninger, A.L., Fiskum, G., Vercesi, A. & Tew, W. in *Calcium and Phosphate Transport across Biomembranes* (eds Bronner, F. & Peterlik, M.) 73-78 (1981).
12. Fry, C.H., Powell, T., Twist, V.W. & Ward, J.P.T. *Proc. R. Soc. B* (in the press).
13. Harrison, S.M. & Miller, D.J. *J. Physiol., Lond.* **353**, 55P (1984).
14. Fry, C.H. & Miller, D.J. in *Control and Manipulation of Calcium Movement* (ed. Parratt, J.) (Raven, New York, in the press).

No pattern yet for snowflakes

SIR — It surprises me that John Maddox's *News and Views* article¹ makes no reference to the role of electric forces in determining the structure of snowflakes. Admittedly, short-range forces are implied by the surface tension effects considered but long-range forces are also likely to have an

effect. One would expect that with a material of such high polarizability as water (dielectric constant 78) the electric field around charged ice crystals gathers water molecules at a far higher rate than would result from the random walks of neutral particles. The ice crystals will be charged by photoelectric emission, most of the ejected electrons being collected by water molecules. The photo-energy will be used in transporting the polarized molecules by electric attraction.

The initial ice crystals are long hexagonal prisms and growth will take place on the major faces, under suitable conditions, thus causing the hexagonal symmetry of the snowflakes. Electrical mutual repulsion between growths on separate faces will prevent them from coalescing to merely increase the growth of the nucleating prism. Possibly these crystals formed in darkness, uncharged, by the random walk process. There are many factors likely to influence the form of growth in addition to the very obvious; temperature and water content of the atmosphere. Air pollution of various kinds, turbulence and electric fields of large scale such as those which give rise to lightning may all have such effects. There is some evidence that long ice crystals may be aligned vertically by an electric field, under calm conditions^{2,3}.

It is unclear to me why there should be any need to invoke lattice vibrations to "tell one growing face what the shape of the opposite growing face is like"¹. It does not have 'the need to know'. In a statistically uniform environment of many particles, statistics and electric fields will shape all six growths uniformly.

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1. Maddox, J. *Nature* **313**, 93 (1985).
2. Mortley, W.S. *New Scientist*. 5 July, 47 (1979).
3. Watts B.C. *New Scientist*. 19 July, 227 (1979).

SIR — The missing element for models of snowflake growth seems to be some property which could coordinate growth across a crystal. May I suggest that the unneutralized partial charges at the surface of the crystal may provide such a mechanism? In the case of proteins, unneutralized charge at the surface generates electrostatic fields through the interior of the molecule which affect protein structure elsewhere in the molecule. In the case of snowflakes, possibly comparable electrostatic fields could provide an attractive force at the minor axis, thus leading to greater crystal growth at the smaller diameters. Whether such a mechanism could account for crystal symmetry would depend on details of charge structure at the surface of the crystal and the permittivity of the interior.

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Parasegments and compartments in the *Drosophila* embryo

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*One of the first steps in segmentation of the *Drosophila* embryo seems to be not the formation of segments, but instead the definition of 14 domains, each of which encroaches into adjacent segments. We call these domains parasegments and discuss their developmental significance.*

But when the morphologist compares one animal with another, point by point or character by character, these are too often the mere outcome of artificial dissection and analysis. Rather is the living body one integral and indivisible whole in which we cannot find, when we come to look for it, any strict dividing line even between the head and the body, the muscle and the tendon, the sinew and the bone.

D'Arcy Thompson, *On Growth and Form*
(Cambridge University Press, 1942)

WE now know that this view of an animal is both true and false, at least as far as the fruitfly is concerned. There is good evidence that *Drosophila* is made piecemeal of compartments, yet they are so well fused together that their borders leave no signature in the body pattern^{1,2}. *Drosophila* has traditionally been described as if it is made bit-by-bit, a picture based less on embryology than on morphology, guesswork and even anthropomorphism. One aim of *Drosophila* developmental genetics is to transform this picture from a subjective to an objective one, to understand what Brenner has called the 'internal representation' of the fly³ as distinct from (our) external description. These two descriptions can be at variance; one example is the eye, which to us is a sharply defined organ with a unique function, but in development is only part of the anterior compartment of the antennal segment of the head^{4,5}. At no time in development (except at the very end) is there an eye primordium—a group of identified and committed cells which will give rise to the eye and to nothing else. We know this because late in development a cell can generate a clone of descendants which will form both head cuticle and photoreceptor eye cells. The word 'eye' probably is not written in the fly's internal description.

This problem—that we do not know the rules or understand the principles by which the fly is constructed—has become more evident recently as new results dispute the traditional picture of the young embryo as a series of segmental primordia; these primordia have been described by Poulson⁶ as if they correspond to the segments of the larva and adult. Here, we question this and consider segmentation of the ectoderm and mesoderm from a different viewpoint.

Early segmentation of the ectoderm

The *Drosophila* ectoderm derives from the lateral part of the blastoderm; dorsally only epidermal cells are formed, whereas ventrally, epidermal cells and neuroblasts are intermingled⁷. Early in development, close to the blastoderm stage, the main mass of the ectoderm is divided up into alternating anterior (A) and posterior (P) polyclones which will generate the A and P compartments of the adult (reviewed in ref. 2). One anteriorly-located A plus one posteriorly-located P polyclone constitutes the primordium of a segment⁸. In the adult the A/P compartment boundary extends through the segment and divides appendages into two parts. In the larva the A/P boundary cuts right through the Keilin's organs, sensory structures which are the only evolutionary remnant of the larval legs and are associated closely with the disks that will construct the adult legs^{9,10}. The precise

position of the P/A segment boundary is not so well known, but in the abdominal segments it is near the anterior limit of the denticle bands of the cuticle¹¹. In the fully developed embryo, segments are defined traditionally by structural criteria alone; there is a deep groove in the epidermis at or near the P/A segment boundary and longitudinal muscles spanning the segment attach nearby.

A chain of alternating stripes can be grouped into pairs in two ways. In the ectoderm, segments are AP pairs of compartments and we call the alternative PA pair, where P is the anteriorly located one and A the posteriorly located one, a parasegment (Fig. 1). There are five lines of evidence to suggest that the parasegment is important in the fly's internal representation.

First, there is the pattern of expression of the bithorax complex—an integrated group of selector genes responsible partly for the diversification of compartments (Lewis¹² and reviewed in ref. 13). The *Ultrabithorax* (*Ubx*⁺) function, the best understood element of the bithorax complex, operates mainly in two parasegments (5 and 6; see Fig. 1); this has been shown by genetic experiments^{10,14,15} and supported by the observation that there is a strong band of *Ubx* expression in the sixth parasegment from the blastoderm stage onwards^{16–18}. Studies of larvae deficient for other elements of the bithorax complex indicate that these elements are also deployed in parasegments rather than segments^{10,19}.

Second, there is the activity of the *fushi tarazu* (*ftz*⁺) gene²⁰, one of several pair-rule genes described by Nüsslein-Volhard and Wieschaus²¹. *In situ* hybridizations show that transcription

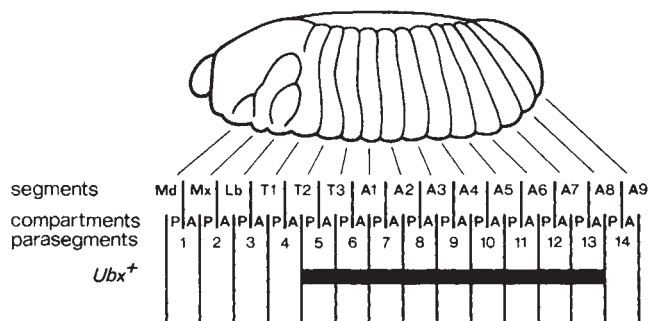


Fig. 1 Expression of *Ubx*⁺ in the *Drosophila* embryo with respect to the different metameric units (parasegments, compartments and segments). Parasegments are one compartment out of register with segments. The pattern of *ftz*⁺ expression²² together with the neuroblast map⁷ suggest that the initially segmented region of the embryo extends from the posterior compartment of the mandibular segment (Md) through the maxillary (Mx), labial (Lb), thoracic (T1–T3) and abdominal (A1–A8) segments to the anterior compartment of A9. The realm of expression of *Ubx*⁺ is as shown, but does not illustrate the differences in degrees of expression within it (see refs 16–18).

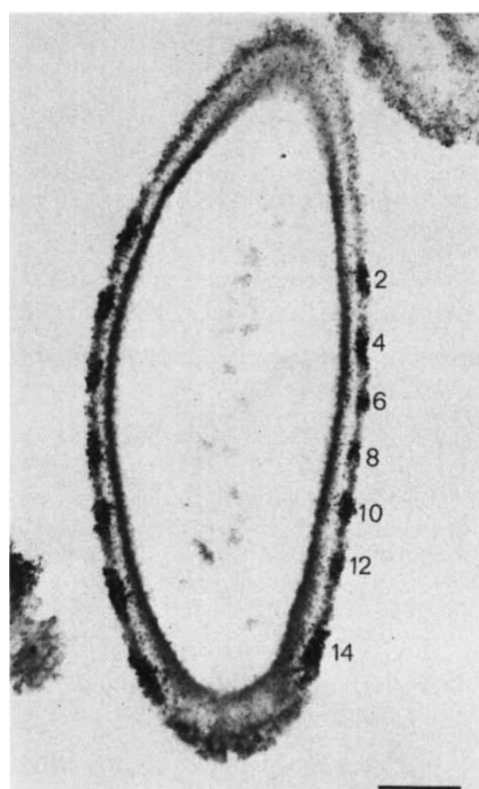


Fig. 2 *In situ* hybridization of *ftz*⁺ double-stranded DNA to a medial section of a wild-type embryo at the late blastoderm stage. Methods and result have been described previously^{16,22,42}. At this stage the *ftz*⁺ RNA can be localized approximately to parasegments 2, 4, 6, 8, 10, 12 and 14; the widths of both labelled and unlabelled bands are about 4 cells²². Scale bar, 50 μ m.

of this gene begins at about the 11th nuclear division; by blastoderm the transcripts have accumulated in seven evenly spaced and sharp stripes, with much less transcript in between²² (Fig. 2). Each stripe is approximately two compartments wide and the registration of the stripes²² suggests they are closer to parasegments than segments. A precise identification of the cells expressing *ftz*⁺ is difficult as the stripes seem to narrow as development proceeds (compare Figs 3b, d and 4b in ref. 22 and our Figs 2 and 5). By the end of germ-band extension, when the first signs of segmentation are visible, the stripes are no longer detectable. The *ftz*⁻ phenotype consists of the elimination, due to cell death, of seven units which are approximately parasegmental^{23,24}. Other pair-rule mutations seem to eliminate similar areas which also span segment boundaries; perhaps the regions removed in *ftz*⁻ are contained in parasegments 2, 4... whereas those missing in *even-skipped* and *hairy* are within parasegments 1, 3... (refs 21, 25).

A third line of evidence refers to the ends of the chain of alternating A and P compartments which extend between the primordia for the fore and hind gut. If the chain contained an integral number of segments it would be expected to begin with the A compartment of the mandibular segment and end on the P compartment of A8 or A9. Alternatively, if the chain consisted of parasegments it should begin with a P compartment and end with an A. The evidence, which is not completely compelling, supports the latter case, as the most anterior compartment in the developing central nervous system seems to be a partial mandibular segment and the most posterior a truncated A9 segment (ref. 7 and M. Bate, unpublished observations). Some independent evidence that there is an A9 segment and that it is incomplete comes from *extra sex comb* (*esc*⁻) embryos where A9 appears as a patch of denticles similar to that normally found only in the anterior compartment of A8 (ref. 26). We therefore

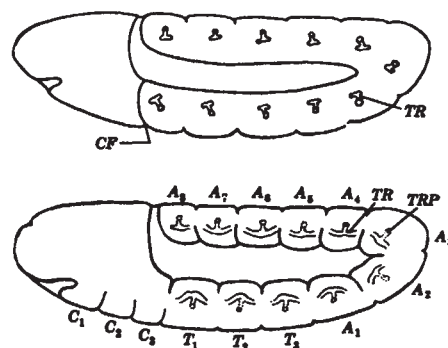


Fig. 3 Reproduction of Poulson's drawing of the early stages of segmentation⁶. In his description of the embryo there are 11 tracheal pits and each one is shown in the middle of each thoracic and abdominal 'segment'; we believe these units are actually parasegments (see text). C₁-C₃ correspond to parasegments 1-3, the three thoracic segments to 4-6 and his eight abdominal segments to parasegments 7-14. Abbreviations: CF, cephalic furrow; TR, trachea; TRP, tracheal pit.

suggest that the most anterior compartment is posterior mandibular, whereas the most posterior one is the anterior compartment of A9. The post-oral epidermis would then consist of 14 parasegments arranged in sequence. Two of the parasegments (numbers 3 and 6) actually span across the traditional subdivision of the insect into head, thorax and abdomen and, of course, in the adult each thoracic leg is formed from parts of two parasegments.

The next evidence concerns the earliest signs of segmentation in the embryo. The key features are reiterated grooves and tracheal pits. Tracheal pits are found in the thorax and abdomen but not in the mouthparts; according to anatomical studies they arise well within zones whose boundaries are visible as grooves on the surface of the embryo²⁷. These zones were described as segments and the grooves as segment boundaries by Poulson (our Fig. 3, from Poulson⁶). But an elegant study by Keilin²⁸, painted a different picture, with the tracheal pits arising not within segments but at the boundary between segments; a picture inferred from his observations that the number of spiracles or tracheal pits in different insect groups is always one less than the total number of segments in the thorax and abdomen. Keilin's view finds strong support from *in situ* hybridization of *Ubx* probes to wild-type embryos¹⁸ and we therefore believe that Keilin's picture is correct and that the grooves described by Poulson (see Turner's pictures, Figs 30 and 32 in ref. 29) are boundaries between the parasegments and not between the segments. These boundaries appear very early, at about 5 h, shortly before the pits develop. We find it significant that the first anatomical subdivisions are not segmental but parasegmental. There is one other problem that the pits raise; Poulson counted 11 of these pits whereas others^{7,27} suggest that only 10 form the tracheal trunk. Sections of embryos show that there is a deep invagination about one segment behind the 10th pit⁷; it is more dorsally located than the other pits but could well be that belonging to the 14th parasegment (Fig. 4a). We believe Keilin's observations²⁸ can be reconciled with those of others if this last pit contributes to the posterior spiracle (Fig. 4a) and this is why it has a somewhat different structure and location.

We conclude that the earliest morphological 'segmental' grooves have been described wrongly and are parasegmental. If the tracheal pits are at the future position of the true segment boundaries, then these boundaries are placed eccentrically in the parasegments (Fig. 4b). Turner and Mahowald's pictures^{27,29} show clearly that the pits are in the anterior third of the parasegment (Fig. 4c), suggesting that in the extended germ band the P compartment is smaller than the A compartment.

Finally, there is evidence from other arthropods that the parasegment may be established very early in development and therefore in evolution. The germ band of certain crustacea is

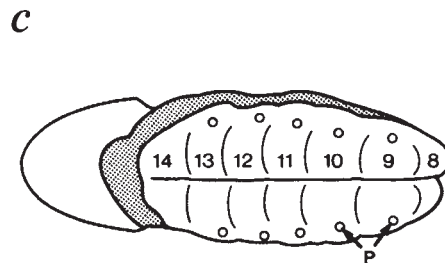
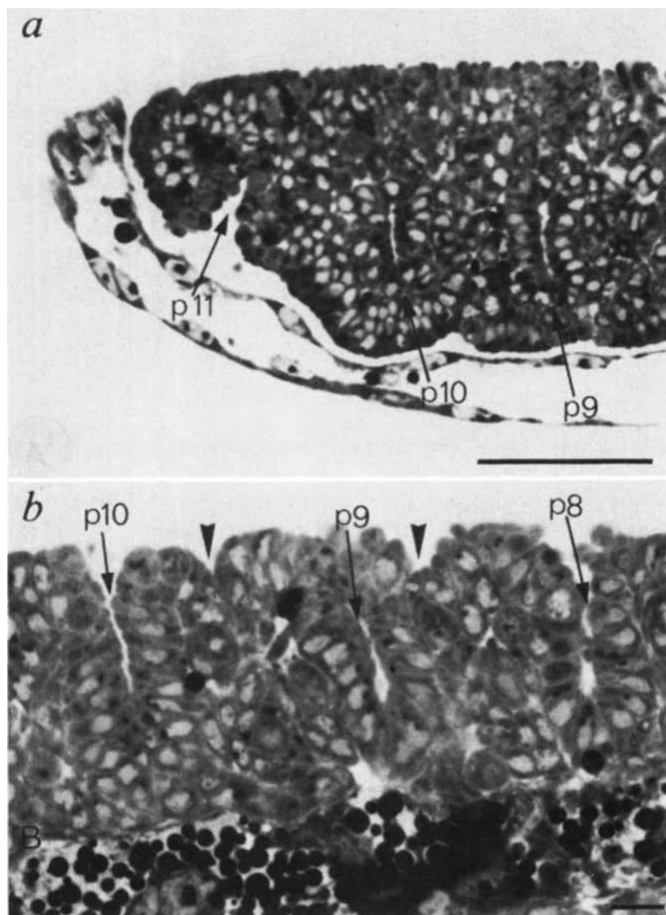


Fig. 4 Relationships between tracheal pits and parasegmental grooves in the *Drosophila* embryo of about 7 h development. *a*, Lateral sagittal section through the ectoderm of parasegments 12–14; note the 11th pit (p11) is different in structure and position from p10 and p9, being more dorsal and cell divisions continuing until germ-band shortening. We believe that the 11th pit contributes to the posterior spiracle. The best evidence for this is the effects of pair-rule mutations; *ftz*, which eliminates the segment boundary contained in parasegment 14, has very rudimentary spiracles, whereas *hairy*, which eliminates the segment boundary in parasegment 13 and leaves that within 14 intact, has normal posterior spiracles. In the embryo the pits are at the segmental boundaries, reflecting the ancestral condition (see Fig. 25 in Keilin's paper²⁸), in the larva and adult they are well within the segments, probably because the tracheae have migrated²⁸. Anterior to the left, ventral to the bottom. Wild-type embryos were fixed as described previously⁴³, embedded in Araldite and sectioned. Scale bar, 50 μ m. *b*, Medial sagittal section of another embryo similar to that shown in *a*. Note parasegmental grooves (arrowheads) are out of register with tracheal pits. Scale bar, 10 μ m. *c*, Tracing of Turner's scanning electron microscope picture of an embryo ~7 h old (ref. 29). Note that the part of the germ band shown (parasegments 14–9) includes that shown in *a*. Parasegmental boundaries appear as grooves medially but not laterally (as also pointed out explicitly in ref. 7). Note the pits (P) seem to be in the anterior part of the parasegment (see text).

produced from lines of cells, each line giving rise to a well-defined parasegmental region including pieces of appendages from neighbouring segments³⁰. Thus, the parasegment but not the segment is represented in the earliest cell lineage of these animals.

Although the case for parasegments as an important unit of insect design could not rest exclusively on any one of these arguments, we find them persuasive when taken together.

Is there counter evidence which suggests that segments are also important units of design in the early embryo? Selector genes in the Antennapedia complex may have their realms of action defined by segment boundaries³¹. *Antennapedia*⁺ has been reported as expressed preferentially within T2 (ref. 32), although the resolution of these studies is not sufficient to eliminate the possibility that the borders of expression are parasegmental. Clonal analysis of the adult and studies of larval phenotypes strongly suggest that the posterior limit of *Sex comb reduced*⁺ requirement is at the P/A segmental border between the P compartment of T1 and the A compartment of T2 (refs 33, 34). So there may also be genes which are expressed in a true segmental fashion, but the limited information we have at the moment points to parasegments and compartments as being first in development and foremost in design.

Segmentation in the mesoderm

The mesoderm arises as a strip of cells along the ventral midline of the blastoderm which invaginates and is soon subdivided into two separate parts, the outer somatic mesoderm and the inner visceral mesoderm^{6,27}. At about the same time (5–6 h of development) as the ectoderm develops shallow grooves (which we have argued are parasegmental boundaries), the mesoderm also becomes organized into cell masses that are demarcated by grooves. By the criterion of their position with respect to the ectodermal grooves and the tracheal pits, the mesodermal masses are in register with the parasegments (our unpublished observa-

tions and see Fig. 31c in ref. 6). Also, the origin of these mesodermal masses can be traced by using the expression of the *ftz* gene as a marker. At blastoderm the *ftz* stripes (Fig. 2) include both presumptive ectoderm and mesoderm³². The straightness of the *ftz* stripes at about 4½ hours (Fig. 5) indicate that the registration of ectoderm and mesoderm is maintained in spite of the cell movements at gastrulation. We therefore believe that the mesodermal masses are parasegmental in origin.

There is evidence that between 5 h and the attachment of muscles to the epidermis at about 14 h, all or part of the mesoderm shifts about one half-segment caudally with respect to the ectoderm so that the mesodermal parasegments are now in register with the ectodermal segments. When *Ubx*⁺ is expressed first in parasegment 6 at the blastoderm stage, the zone of transcription extends continuously into the presumptive mesoderm¹⁸. Later, expression of *Ubx*⁺ in the ectoderm and mesoderm falls out of step so that the strongest peak in the ectoderm is in parasegment 6, which consists of the P compartment of T3 and the A compartment of A1. In the muscles there is expression in A1 but not in T3 (ref. 16). Although this shift could be due to a change in the pattern of transcription, we suggest that it is more probably due to relative cell movement.

Remember that in the ectoderm there is a chain of A and P compartments. Is there a similar chain in the mesoderm? Certainly, clones of cells marked soon after blastoderm can label all the muscles of one segment but do not cross to neighbouring segments, so there are metameric lineage restrictions in the mesoderm, but there is no indication of any A and P compartments in the muscles³⁵. Moreover, the *engrailed*⁺ gene, which is active and required exclusively in P compartments of the epidermis^{36,37}, may well have no role in the mesoderm; cells homozygous for strong *engrailed*-lethal mutations show no phenotype in mesodermal derivatives^{35,38}. These findings suggest, but do not prove, that the mesoderm is subdivided into half as many lineage compartments as the ectoderm and that these lineage compartments are parasegmental in origin.

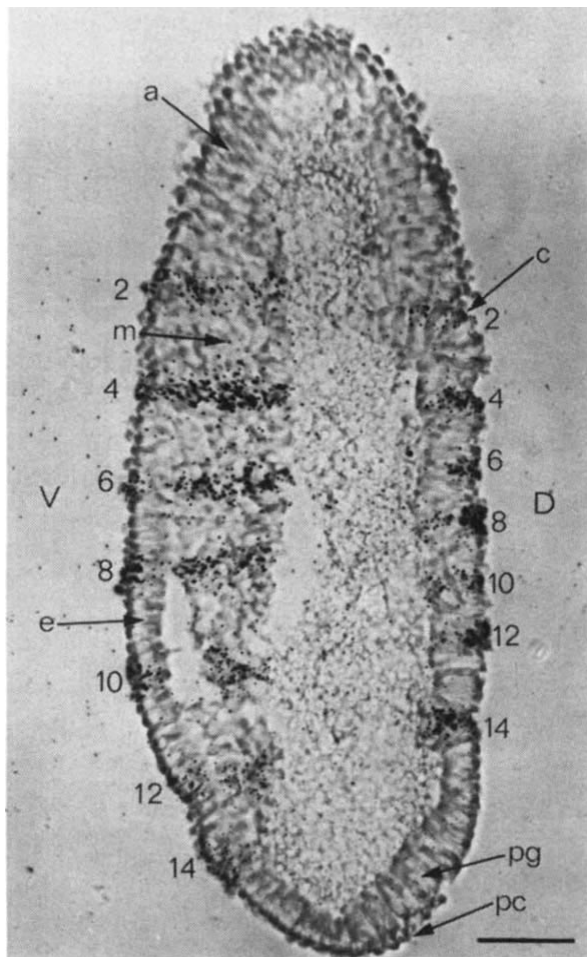


Fig. 5 *In situ* hybridization of *ftz*⁺ DNA to a medial sagittal section of a wild-type embryo at the beginning of germ-band elongation (~4 h). Gastrulation has taken place and the mesoderm lies ventrally above the ectoderm. The seven stripes have narrowed and extend, in register, into the mesoderm. Material prepared as in Fig. 2. a, Anterior midgut; c, cephalic furrow; e, ectoderm; m, mesoderm; pc, pole cells; pg, posterior midgut; D, dorsal; V, ventral. Photograph is a superimposition of two images of the same section; one is phase contrast, the other shows the photographic grains as black dots and is a negative dark-field image. Scale bar, 50 μ m.

Segments

If parasegments and compartments are important in design, what role do segments play in the making of the organism? The main reason why segments have been defined as morphological units in the larva and adult is that the cuticle flexes at the segment boundaries and this is where the longitudinal muscles attach. Segments also seem to be the units of pattern formation; in hemipteran bugs, gradients of positional information appear to reiterate every segment^{8,39} and there is some interruption of cellular communication at the segment boundary^{40,41}. During development, segments can be defined only relatively late; during germ band shortening (~9 h) the grooves defining parasegments deepen considerably and drag in the external remnants

of the tracheal pits (our unpublished observations; Figs 32–36 in ref. 29). Because some 60% of the epidermal surface is drawn into the grooves, it is not clear whether they should be described as segmental or parasegmental at this stage. Later (~11–14 h) the myoblasts fuse and longitudinal muscles attach near to the pit remnants, which are near the segment borders. These muscles reach to the next segment boundary and therefore clearly define segments for the first time. Remember the definition of segments is only descriptive; we do not yet know whether the genome of the fly specifies segments in the same way as it specifies parasegments and compartments. This will probably be elucidated when the patterns of expression of all genes in the Antennapedia complex³¹ and of pair-rule and segment-polarity genes²¹ are described. Segmentation in the pre-oral region remains obscure; the expression of *ftz*⁺ (ref. 22) and the neuroblast map⁷ suggest that it does not segment in the early embryo.

Conclusions

The picture of segmentation in the ectoderm and mesoderm is slowly sharpening. By the objective criteria of cell lineage and the expression of genes in the bithorax complex and by the more subjective criteria of morphology, it seems that parasegments are fundamental units of pattern formation in the young embryo; they may be the only metameric unit in the mesoderm. Many questions remain unanswered; for example, we do not know if the embryo is first divided into larger domains and then into parasegments and compartments, nor do we know if, in the ectoderm, parasegments are replaced by segments as units of design as development proceeds.

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- Garcia-Bellido, A., Ripoll, P. & Morata, G. *Nature* **245**, 251–253 (1973).
- Garcia-Bellido, A., Lawrence, P. A. & Morata, G. *Scient. Am.* **241**, 102–110 (1979).
- Brenner, S. in *Cellular Controls in Differentiation* (eds. Lloyd, C. W. & Rees, D. A.) 3–7 (Academic, New York, 1981).
- Morata, G. & Lawrence, P. A. *Nature* **274**, 473–474 (1978).
- Morata, G. & Lawrence, P. A. *Dev. Biol.* **70**, 355–371 (1979).
- Poulson, D. F. in *The Genetics and Biology of Drosophila* (ed. Demerec, M.) 168–274 (Wiley, New York, 1950).
- Hartenstein, V. & Campos-Ortega, J. A. *Wilhelm Roux Arch. dev. Biol.* **193**, 308–325 (1984).
- Lawrence, P. A. *Cell* **26**, 3–10 (1981).
- Keilin, D. *Bull. scient. Fr. Belg.* **49**, 1–198 (1915).
- Struhl, G. *Nature* **308**, 454–457 (1984).
- Szabad, J., Schüpbach, T. & Wieschaus, E. *Dev. Biol.* **73**, 256–271 (1979).
- Lewis, E. B. *Nature* **276**, 565–570 (1978).
- Lawrence, P. A. & Morata, G. *Cell* **35**, 595–601 (1983).
- Morata, G. & Kerridge, S. *Nature* **290**, 778–781 (1981).
- Hayes, P. H., Sato, T. & Denell, R. E. *Proc. natn. Acad. Sci. U.S.A.* **81**, 545–549 (1984).
- Akam, M. E. *EMBO J.* **2**, 2075–2084 (1983).
- White, R. & Wilcox, M. E. (1984) *Cell* **39**, 163–171 (1984).
- Akam, M. E. & Martinez-Arias, A. *EMBO J.* (submitted).
- Morata, G., Botas, J., Kerridge, S. & Struhl, G. *J. Embryol. exp. Morph.* **78**, 319–341 (1983).
- Wakimoto, B. T. & Kaufman, T. C. *Dev. Biol.* **81**, 51–64 (1981).
- Nüsslein-Volhard, C. & Wieschaus, E. *Nature* **287**, 795–801 (1980).
- Hafen, E., Kuroiwa, A. & Gehring, W. J. *Cell* **37**, 833–841 (1984).
- Nüsslein-Volhard, C., Wieschaus, E. & Jürgens, G. *Verh. dt. zool. Ges.* 91–104 (1982).
- Ingham, P. W. & Martinez-Arias, A. *Nature* (submitted).
- Sander, K., Lohs-Schardin, M. & Baumann, M. *Nature* **287**, 841–843 (1980).
- Struhl, G. *Nature* **293**, 36–41 (1981).
- Turner, F. R. & Mahowald, A. P. *Dev. Biol.* **57**, 403–416 (1977).
- Keilin, D. *Parasitology* **36**, 1–66 (1944).
- Fullilove, S. L. & Jacobson, A. G. in *Genetics and Biology of Drosophila* Vol. 2c (eds Ashburner, M. & Wright, T. R. F.) 105–127 (Academic, London, 1978).
- Dohle, W. *Zoomorphologie* **84**, 235–277 (1976).
- Kaufman, T. C. in *Time, Space and Pattern in Embryonic Development*, 365–383 (Liss, New York, 1983).
- Levine, M., Hafen, E., Garber, R. L. & Gehring, W. J. *EMBO J.* **2**, 2037–2046 (1983).
- Struhl, G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7380–7384 (1982).
- Struhl, G. *J. Embryol. exp. Morph.* **76**, 297–331 (1983).
- Lawrence, P. A. *Cell* **29**, 493–503 (1982).
- Morata, G. & Lawrence, P. A. *Nature* **255**, 614–617 (1975).
- Kornberg, T. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1095–1099 (1981).
- Lawrence, P. A. & Johnston, P. *EMBO J.* **3**, 2839–2844 (1984).
- Locke, M. J. *exp. Biol.* **36**, 459–477 (1959).
- Warner, A. E. & Lawrence, P. A. *Cell* **28**, 243–252 (1982).
- Blennhasset, M. G. & Caveney, S. *Nature* **309**, 361–364 (1984).
- Hafen, E., Levine, M., Garber, R. L. & Gehring, W. J. *EMBO J.* **2**, 617–623 (1983).
- Zalokar, M. & Erk, I. *Stain Technol.* **52**, 89–95 (1977).

Solidification and recharge of SiO₂-rich plutonic magma chambers

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There exists substantial variation in initial strontium isotopic composition in rocks from three otherwise homogeneous tonalite plutons. We interpret disrupted dykes as conduits through which heterogeneous liquids were added periodically to the inflating magma chambers. Recharge buffered the physicochemical but not the strontium isotope properties of the crystallizing liquids.

AN understanding of the origin of the great continental margin batholiths of the world is fundamental to the development of theories on the nature and origin of the continental crust. The magmas ultimately solidifying to produce the batholiths, however, may be highly modified from those of the original magmas formed at depth in the crust or upper mantle. These changes occur by various processes acting both during melt collection and transport, as well as during magma chamber inflation and crystallization. Recent theoretical, experimental and geological observations suggest that the processes operating in a large crustal magma chamber are considerably more complex than has generally been thought¹⁻⁵.

We have therefore studied in detail three large, well-exposed tonalitic plutons (bodies of intermediate composition granular igneous rocks) in the San Jacinto Mountains of southern California (Fig. 1) and have developed a model for the filling and crystallization of these relatively SiO₂-rich crustal magma chambers, involving periodic recharge of a fractionating system with new isotopically-variable magma pulses. This recharge-fractional crystallization process buffered the liquids in the magma chambers thermally and chemically (but not isotopically) throughout much of their crystallization histories, yielding large volumes of mineralogically and chemically homogeneous, but isotopically heterogeneous, tonalite.

Geological relationships

Rocks from the Peninsular Ranges batholith (PRB) were studied. The PRB developed during a single Cretaceous magmatic cycle and shows regular transverse (west-east) asymmetries⁶⁻¹⁰. More than two distinct end-member compositions are involved in the origin of the bulk of the batholith⁶⁻⁸ and a further component (possibly old continental lithosphere) is required for the San Jacinto rocks^{6,11}.

Three large and many small plutons outcrop in the San Jacinto Mountains (Fig. 1). Three large tonalite masses were emplaced into an heterogeneous assemblage of slightly older igneous, and metasedimentary rocks. Each major tonalite spans a limited compositional range from mafic tonalite (colour index (CI) > 15) to K-feldspar-poor granodiorite (CI ~ 10). Minor felsic differentiates extend the compositional range to granodiorite. Units I and II are slightly more mafic (that is, more hornblende- and biotite-rich) than Unit III. All units are comprised of plagioclase, of composition anorthite (An₃₀₋₄₀) (50-55 volume %), quartz (20-30%), K-feldspar (1-8%), biotite (10-15%), hornblende (0-5%), titanite (0-2%) and accessory zircon, apatite, allanite and ilmenite. Variations in mineral abundances are geographically systematic only in Unit III, which grades from marginal mafic tonalite to central K-feldspar-poor granodiorite. Alignment of foliations, lineations and inclusions (Fig. 1) and apparent flow-sorting and scour features seem to reflect magmatic flow patterns in each chamber (R.I.H., unpublished data).

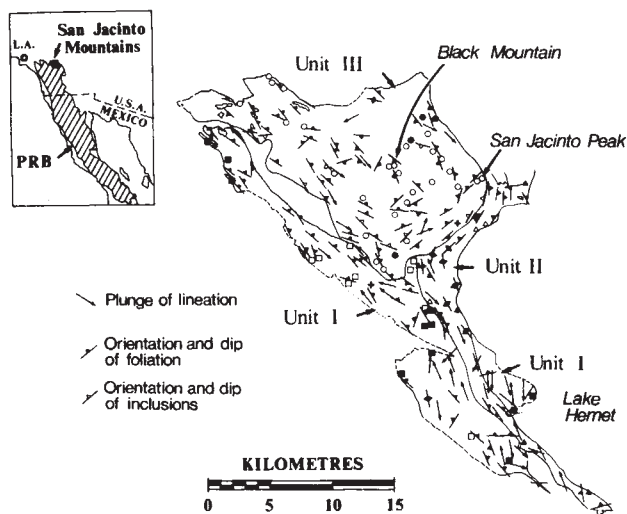


Fig. 1 Simplified geological map (palinspastic base) showing structural data and sample localities for the three major tonalite units in the San Jacinto Mountains. Inset shows location at the northern end of the PRB of southern and Baja California. Unit II was emplaced before complete solidification of Unit I; Unit III, in turn, was emplaced before complete solidification of Unit II. Conservative depth extrapolations imply that both Units I and III have total volumes > 1,000 km³. Solid symbols are for samples with SiO₂ < 65.5 wt% and open symbols for other tonalites and K-feldspar-granodiorites (SiO₂ > 65.5 wt%). Unit I, ■, □; Unit II, ▲, △; Unit III, ●, ○.

The three major tonalites yield zircon U-Th-Pb crystallization ages in a narrow interval from 93 to 94 Myr; two studied older intrusives are at most 4-5 Myr older, implying that the entire igneous assemblage was emplaced and solidified in at most 6 Myr (L.T.S. and R.I.H., unpublished data).

Mineral compositions in the three major tonalites are remarkably uniform. The An content of the bulk plagioclase calculated from mineral and rock chemical data falls from An₄₀ in the most mafic tonalites to An₃₀ in the K-feldspar-poor granodiorites; Mg/(Mg + Fe) of biotite and hornblende (determined by electron microprobe) drop similarly from 0.44 to 0.36. The homogeneity of mineral compositions implies remarkable stability of physicochemical conditions throughout solidification of each unit.

Unit III is the best preserved and best exposed of the three major plutons and is considered here in two parts. In the northwestern portion, foliations are generally shallow with random orientations. Small patches of both metasedimentary and igneous country rock are present; we suggest that this part of the pluton is generally within a few hundred metres of the

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original pluton roof. The eastern two-thirds of Unit III is exposed currently at a level 1–3 km beneath the inferred pluton roof. Foliations here define a concentric pattern centred near Black Mountain (Fig. 1). Foliation intensity and dip both decrease (dip from ~ 70 to $\sim 10^\circ$) inwards from the pluton margin, giving an overall resemblance to a series of systematically stacked funnels which successively shallow upwards.

Compositions of rocks from Unit III also change systematically inward. Marginal rocks are mafic tonalites, commonly heterogeneous at the centimetre-metre scale, with abundant schlieren, layering and inclusions. Rocks free of such effects may vary considerably in colour index ($15 < CI < 25$). Marginal tonalites commonly contain $>5\%$ hornblende and 1–3% K-feldspar. Mafic mineral abundances decrease (to $CI \sim 10$) and K-feldspar abundances increase (to $\sim 8\%$) away from contacts, so that rocks from around Black Mountain are K-feldspar-poor granodiorites containing only minor hornblende. Inclusion and schlieren abundance also decrease away from the pluton margins, so that the inner third (by area) of the eastern portion of Unit III is composed of relatively featureless felsic tonalite and K-feldspar-poor granodiorite containing uncommon inclusions and inclusion trains and cut by rare mafic dykes.

Inclusions, inclusion trains and dykes

Ellipsoidal dark inclusions with maximum dimensions of ~ 1 m are common throughout the more mafic tonalites, locally comprising over 50% of the rock. They are aligned usually in the foliation plane defined by oriented mafic minerals in the host tonalite. The most extensive tabular concentration of inclusions, or inclusion train, forms the outermost part of the eastern margin of Unit III. This train (or composite of several trains) is ≤ 200 m wide and is continuous for 27 km. Inclusion trains often can be traced for 0.1 to several kilometres before they die out or exposure is lost. Such trains are less common with increasing distance from pluton contacts; they are rare or absent from the central part of Unit III.

Dark inclusions contain the same minerals (except K-feldspar) as the host tonalites, but some have different proportions; hornblende is commonly much more abundant and biotite less abundant compared with host tonalites. Mineral compositions are approximately similar; $Mg/(Mg + Fe)$ of mafic minerals and An content of plagioclase are marginally higher (by a few mole %) in inclusions. Inclusions commonly contain relatively large (to a few millimetres) euhedral to subhedral plagioclase tablets, smaller euhedral to subhedral hornblende prisms and large rounded equant quartz grains forming an interlocking framework filled in by smaller and less well-formed plagioclase, hornblende and quartz crystals, with irregular biotite and anhedral titanite. The largest plagioclase and hornblende crystals appear as distinct phenocrysts in hand specimen. Rare plagioclase tablets contain overgrown cores, suggesting a complex history. Plagioclase, hornblende and quartz seem to have been the early crystallizing phases.

Accidental xenoliths of country rock and cognate inclusions are also found rarely. Cognate inclusions (early solidifying rocks from the pluton margins re-incorporated into the magma) are similar texturally to host tonalites, lack phenocrystic plagioclase and are recognized most easily when fragments are derived from nearby scoured or slumped pluton walls.

Fine-grained dark dykes, ~ 10 cm to 2 m in width and up to several kilometres in length, intrude the tonalites and are texturally and mineralogically similar to the common inclusions. Mutual veining of dyke and host is common; pegmatoidal patches adjacent to contacts and apparent shear of dyke margins into tonalite are found also. The dominant rock type in the marginal inclusion train of Unit III is dyke-rock in all stages of veining by tonalite. Dykes in all stages of intrusion and subsequent break-up by injection of tonalite are present; the end result of this process is an assortment of randomly oriented blocks of dyke material in a tonalitic matrix. Similar relations have been described from the Sierra Nevada batholith^{12,13}. There

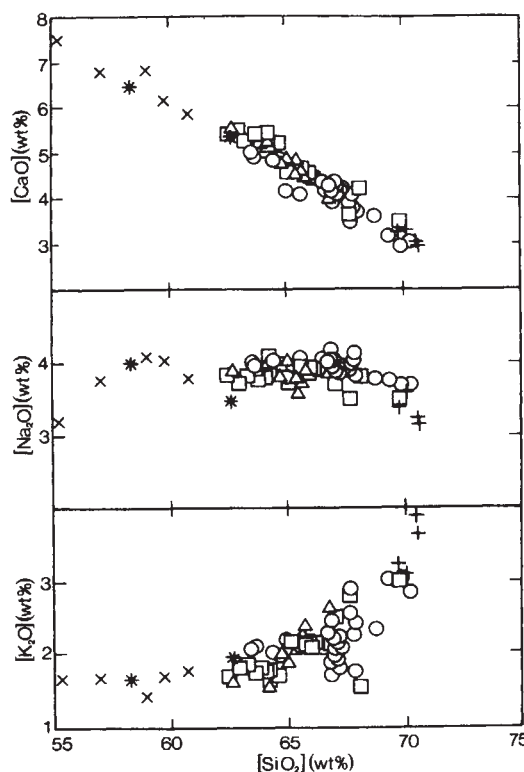


Fig. 2 Harker diagrams for CaO, Na₂O and K₂O. Symbols as Fig. 1 with: +, felsic differentiates; x, mafic inclusions; *, dyke-rocks.

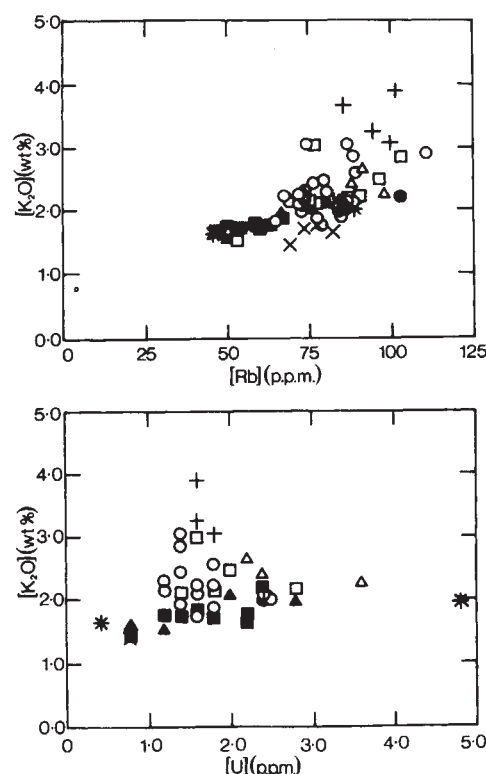


Fig. 3 K-Rb and K-U diagrams for major tonalites. Symbols as for Figs 1 and 2. Mafic tonalites show linear element-element relations in contrast to the more complex behaviour shown by more-felsic rocks. These patterns are interpreted as resulting from mixing of simply related but heterogeneous new mafic liquids with fractionated liquids (represented by the felsic differentiates) in the magma chambers. Tonalite compositions are not representative of liquid compositions, unlike inclusion and dyke-rock compositions.

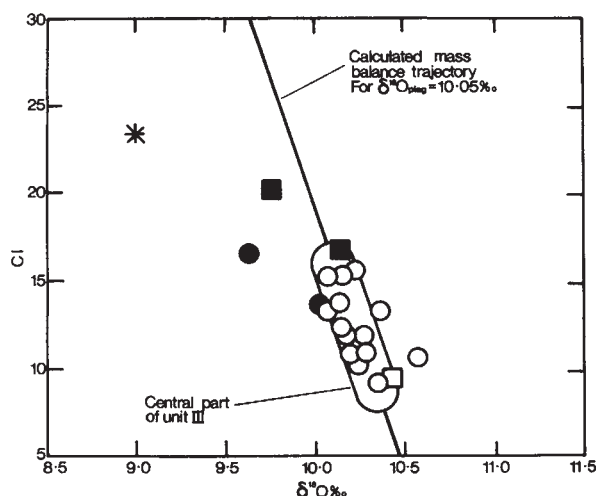


Fig. 4 CI plotted against $\delta^{18}\text{O}$ for unexchanged rocks. A calculated mass-balance trajectory is shown; variations in hornblende/biotite and quartz/feldspar ratios give a range of $\pm 0.15\%$ in the $\delta^{18}\text{O}$ value calculated for a given CI, but do not alter substantially the slope of the line. Symbols as for Figs 1 and 2.

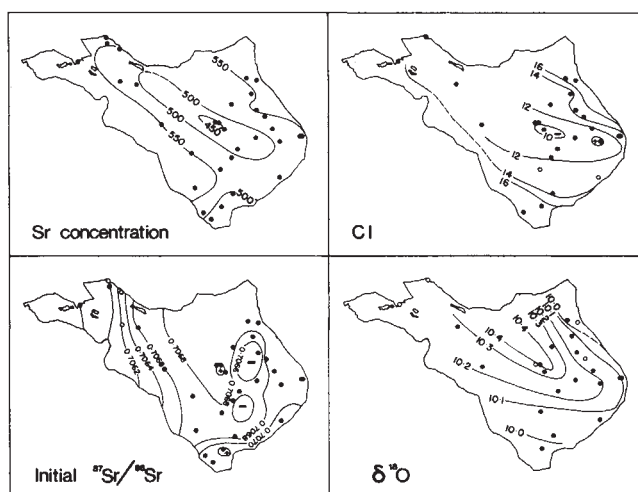


Fig. 5 Geographic variation in Sr concentration (p.p.m.), Sr_i (volume %), and $\delta^{18}\text{O}$ (‰) for Unit III. Open symbols for Sr_i and $\delta^{18}\text{O}$ represent samples with either petrographical or oxygen isotope evidence for substantial exchange with country-rock fluids and are not used in the contouring. Open symbols for CI are for samples not representative of the bulk of the rock in the vicinity and are also not used in contouring.

is a complete gradation in outcrop from dykes through broken-up dykes to inclusion trains. We suggest that many of the inclusion trains in these plutons, and, by inference, individual inclusions distributed throughout the various intrusive bodies studied, are derived principally from disaggregated dykes or pillows of mafic liquid injected into a mass of partially liquid tonalite in the slowly-solidifying magma chambers. Observations in well-exposed areas suggest that dykes with a composition similar to that of the host tonalites are more abundant than are mafic (quartz-dioritic) dykes. The lack of contrast between dyke and host material, however, makes identification (and thus quantification) possible only in exceptional circumstances. The dykes were the conduits through which substantial amounts of material were added to the inflating magma chambers. Flow through a single fissure 1 m wide and 1 km long at a velocity of 10^{-3} m s^{-1} could fill Unit III in 10^4 – 10^5 yr. Flow would probably be intermittent as individual fissures open and close.

A range of features could be developed during the addition of each new input, depending on the thermal and chemical contrasts between the new and pre-existing liquids in a pluton. Many of the planar features common in large SiO_2 -rich plutons (schlieren, compositional contrasts) may owe their origin, at least in part, to processes operating during the emplacement of new liquids into pre-existing magma chambers.

Geochemical and isotopic results

The three tonalite units overlap in both chemical and isotopic compositions, except that Unit III is both slightly more felsic

and more homogeneous than either Unit I or II (Table 1). The ranges for SiO_2 and K_2O listed here are biased by the inclusion of volumetrically insignificant 'felsic granodioritic differentiates' collected to sample extreme variations. The homogeneity of the three units is shown on the Harker diagrams (Fig. 2); 60% of samples from Unit III have 66.5–68.0 wt% SiO_2 and 70% of samples from Units I and II have 62.5–65.5 wt% SiO_2 , consistent with field observations that Units I and II are generally a little more mafic than Unit III. Rocks in these restricted compositional ranges span almost the entire observed range of initial $^{87}\text{Sr}/^{86}\text{Sr}$ (Sr_i). Major elements (except K) and Sr, Zr, Sc, Ga, V, Cr and Zn give tight linear arrays on Harker diagrams (Fig. 2). Inclusion and dyke samples fall on the low SiO_2 extrapolation of these trends (except for Sr, Zr and Cr). Elements relatively high in the felsic differentiates (K, Ba, Rb, U, Th, Pb and rare-earth elements) have more complex element–element relations. Behaviour of K–U and K–Rb (Fig. 3) are typical; analyses fall in a triangular field, with the mafic tonalites (defined arbitrarily as those with $\text{SiO}_2 < 65.5$ wt%) defining the base of the triangle and the felsic differentiates forming the apex.

Except in the most mafic rocks, which have variable $\delta^{18}\text{O}$ values, $\delta^{18}\text{O}$ shows a narrow range that correlates with rock composition (Figs 4 and 5), whereas Sr_i does not (Fig. 5). The steady increase of $\delta^{18}\text{O}$ from +10.0 to +10.4‰ (Fig. 4) results from the smaller proportion of the low ^{18}O minerals hornblende and biotite and a larger proportion of relatively high ^{18}O K-feldspar in more felsic central rocks. All but the two most mafic rocks from Unit III could have solidified from a homogeneous

Table 1 Chemical and isotopic data for rock samples from major tonalite bodies

Name	No. of samples analysed	SiO_2 (wt%)		K_2O (wt%)		Rb (p.p.m.)	Sr (p.p.m.)	Sr_i	$^{18}\text{O}^*$
		Mean	Range	Mean	Range				
Unit I	22	65.5	62.5–70.1	2.2	1.64–3.06	48–99	430–618	0.7060–0.7077	+9.8 to +10.4
Unit II	10	65.5	62.7–70.5	2.2	1.55–3.89	48–97	407–620	0.7058–0.7074	9.7†
Unit III	30	66.8	63.5–70.6	2.3	1.75–3.64	62–106	439–588	0.7058–0.7073	+9.6 to +10.6
Inclusions	5	—	55.4–60.8	—	1.45–1.79	48–79	499–671	0.7068–0.7073	—
Dyke-rock	2	—	58.4, 62.9	—	1.65, 1.97	44, 80	787, 432	0.7084, 0.7072	—, +9.0

Chemical analysis by X-ray fluorescence^{21,22}; $^{87}\text{Sr}/^{86}\text{Sr}$ at time of crystallization (Sr_i) calculated using the mean zircon U–Pb age for the intrusives (L.T.S. and R.I.H., unpublished data) and is referenced to a value of 0.70800 for E&A SrCO_3 . Oxygen isotope data are reported in the standard δ -notation²³ relative to a value of 0.0 for SMOW. NBS-28 has $\delta^{18}\text{O} = +9.60$ on this scale. *, $\delta^{18}\text{O}$ data available for a more limited population; †, single sample.

(for oxygen) liquid with a $\delta^{18}\text{O}$ value of approximately +10.2. The two more mafic samples ($\delta^{18}\text{O}=9.0, 9.6$) could not have precipitated from this liquid and indicate at least some initial oxygen isotope heterogeneity in this pluton. The sample with $\delta^{18}\text{O}=+9.0\%$ is a dyke-rock texturally similar to unanalysed host tonalites.

In contrast to the $^{18}\text{O}/^{16}\text{O}$ data, the strontium isotope data can only be interpreted as preserving (perhaps modified) significant initial liquid heterogeneity. Sr_i serves as a tracer of original liquid properties unaffected by magma chamber processes; the sparse dyke and inclusion data demonstrate the presence of liquids during pluton inflation and solidification with a range in Sr_i from at least 0.7068 to 0.7084 (Table 1).

Conclusions

Our data show that each of the major plutons is homogeneous in major element composition and mineral chemistry. Unit III shows some concentric zonation for both major and trace elements and for $\delta^{18}\text{O}$. Sr_i , however, is quite variable in each pluton and does not correlate well with any other measured parameter. These observations place constraints on the evolution of the magma chambers that produced these plutons.

The Sr_i variations are not compatible with solidification of these rocks from large, closed-system magma chambers; open-system behaviour of some type is inferred. We conclude that dykes represent conduits through which new material was added periodically to each chamber, providing a mechanism for a more dynamic system. The chemical relations result from mixing of new, relatively mafic liquids (with an average composition of tonalite) with slightly more-evolved liquids already present in the magma chambers. The most extreme of these liquids are represented by the analyses of the uncommon felsic differentiates. All analysed samples can be interpreted simply as mixtures of such felsic evolved liquids and liquids with a compositional range encompassing at least the inclusion and mafic tonalite compositions. Unless there has been major loss of material either upward or downward from the 3 km of vertical exposure, the average composition of added liquid is simply the bulk composition of each pluton (Table 1). Relatively SiO_2 -rich values (in comparison with mafic dyke and inclusion values) are consistent with field observations that the most abundant dykes have tonalitic compositions.

Once a horizontally extensive magma chamber had developed, it intercepted and incorporated all liquids rising from deeper source regions that rose as far as its base. This is analogous to the eruption of basaltic magmas outside the diameter of an extensive molten rhyolite sheet in rhyolitic caldera environments but not within the caldera itself, where they are trapped beneath the low-density rhyolite magma¹⁴⁻¹⁷.

The effects of replenishment will depend on the frequency, relative volume and chemical and isotopic properties of each liquid increment, which cannot be constrained tightly at present. Some deductions can be made from the observed relationships: (1) The extent of fractionation between successive increments must have been small compared with the volume of the chamber, otherwise more extensive variation in rock and mineral chemistry would be expected. (2) Small influxes of hotter new magma would be cooled quickly to the ambient temperature of the chamber. Crystallization would produce inevitably both

crystals and a liquid differing little in major element and probably trace element composition from the surrounding melt¹⁸. Both crystals and hybrid liquid could, however, differ in various isotopic parameters from those of the surrounding melt. Repeated replenishment tends to buffer both the composition and the temperature of the liquid in the chamber¹. If replenishment and solidification are approximately in balance, large volumes of homogeneous solids necessarily result, the composition of which relate directly to the composition of the added liquids, as must occur for Units I, II and the outer rocks of Unit III. If rapid inflation of a magma chamber is followed by cessation of the replenishment system, closed system fractionation may develop as has occurred for the inner part of Unit III. The extent of fractionation is dependent on liquid composition. (3) The discordances and rapid local variations in Sr_i necessitate considerable isotopic variability in the added liquids, consistent with observations that the dykes and inclusions, inferred to represent samples of these added liquids, show such variation. The isotopic composition of a rock at a particular location is thus determined by the isotopic composition of the particular batch (or batches) of liquid from which it crystallized; the isotopic composition of added liquids is itself a function of source composition, shown for the PRB to be strongly geographically variable⁶⁻⁹. (4) The variability of Sr_i in the central part of Unit III, where recharge had apparently either ceased or become insignificant, implies that the liquid remaining in the magma chamber was not stirred efficiently at the scale of ≥ 1 km. This could result from increasing yield strength in the cooling liquids inhibiting convection or from the development of localized convection patterns.

Our interpretation of the bulk of the San Jacinto mafic inclusions as samples of quenched magmas injected into the inflating plutons has more widespread applicability to the problem of the origin of the mafic inclusions common in granitoid rocks. Evidence has been cited supporting an origin for the common granitoid inclusions (enclaves) by magma mixing (reviewed in ref. 19); the inclusions may form by mingling and quenching of globules of more mafic magma in the host granitoid magma. We do not recognize a restite-derived inclusion population at San Jacinto, compatible with the inferred high temperature and relatively low viscosity of these magmas (R.I.H., unpublished data). This contrasts with the abundant restite of the lower temperature, more viscous plutons of southeastern Australia²⁰. Inclusions in granitoids will have a wide variety of origins dependent on the details of magma production and transport, as well as on the mechanisms of pluton emplacement and solidification.

We conclude that the crystallization behaviour of the San Jacinto magma chambers was controlled by a recharge-fractional crystallization process. Operation of such a process buffers the chemical and thermal properties of solidifying magmas and may yield vast volumes of geochemically and mineralogically homogeneous but isotopically heterogeneous rock.

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- O'Hara, M. J. *Nature* **266**, 503-507 (1977).
- McBirney, A. J. & Noyes, R. M. *J. Petrol.* **20**, 487-554 (1979).
- McBirney, A. J. *J. volcan. geotherm. Res.* **7**, 357-371 (1980).
- Huppert, H. E. & Turner, J. S. *J. Fluid Mech.* **106**, 299-329 (1981).
- Hildreth, W. *J. geophys. Res.* **86**, 10153-10192 (1981).
- Taylor, H. P. Jr & Silver, L. T. *U.S. Geol. Surv. Open-File Rep.* **78-701**, 423-426 (1978).
- Silver, L. T., Taylor, H. P. Jr & Chappell, B. W. in *Mesozoic Crystalline Rocks* (eds Abbott, P. L. & Todd, V. R.) 83-110 (Dept Geol. Sciences, San Diego State University, 1979).
- DePaolo, D. J. *J. geophys. Res.* **86**, 10470-10488 (1981).
- Silver, L. T., Early, T. O. & Anderson, T. H. *Geol. Soc. Am. Abstr.* **15**, 375 (1975).
- Gromet, L. P. & Silver, L. T. *Geol. Soc. Am. Abstr.* **11**, 436 (1979).
- Hill, R. I., Silver, L. T. & Taylor, H. P. Jr *Contrib. Miner. Petrol.* (submitted).

- Compton, R. R. *Manual of Field Geology* (Wiley, New York, 1962).
- Reid, J. B. Jr, Evans, O. C. & Fates, D. G. *Earth planet. Sci. Lett.* **66**, 243-261 (1983).
- Christiansen, R. L. & Blank, H. R. Jr *U.S. Geol. Surv. Prof. Pap.* **729B**, (1972).
- Bailey, R. A., Dalrymple, G. B. & Lanphere, M. A. *J. geophys. Res.* **81**, 725-744 (1976).
- Robinson, H. H. *U.S. Geol. Surv. Prof. Pap.* **76**, (1913).
- Moore, R. B. & Wolfe, E. W. *U.S. Geol. Surv. Misc. Invest. Map* **1-953** (1976).
- Sigurdsson, H. & Sparks, R. S. J. *J. Petrol.* **22**, 41-84 (1981).
- Vernon, R. H. *J. Proc. R. Soc. N.S.W.* **116**, 77-103 (1983).
- Compton, W. & Chappell, B. W. in *The Earth: Its Origin, Structure and Evolution* 377-426 (Academic, London, 1979).
- Norrish, K. & Hutton, J. T. *Geochim. cosmochim. Acta* **33**, 431-453 (1969).
- Norrish, K. & Chappell, B. W. in *Physical Methods in Determinative Mineralogy*, 2nd edn, (ed. Zussman, J.) 201-272 (Academic, London, 1977).
- Taylor, H. P. Jr & Epstein, S. E. *Bull. geol. Soc. Am.* **73**, 461-480 (1962).

Rearrangement and transcription of the β -chain genes of the T-cell antigen receptor in different types of murine lymphocytes

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Rearrangements of T-cell receptor β -chain genes are usually found on both chromosomal homologues, occurring by both deletional and non-deletional mechanisms. Two constant-region (C_β) genes have been identified previously and at least one is transcribed in every helper or cytotoxic T cell tested, but the choice of C_β gene expression is not correlated with the specialized functions of these T lymphocytes. By contrast, four of five suppressor T-cell hybridomas examined have deleted all known joining (J_β) gene segments and C_β genes and therefore may have antigen receptors encoded by different T-cell receptor gene families.

THERE are at least three separate functional classes of T lymphocytes: helper T cells stimulate immune responses, suppressor T cells diminish these responses and cytotoxic T lymphocytes kill cells expressing antigenic molecules. T cells bind and respond to antigen only in the presence of specific gene products encoded by the major histocompatibility complex (MHC)^{1,2}. The functional class of a given T cell is correlated with the recognition of particular MHC-encoded molecules. Most helper T cells recognize antigen in conjunction with the class II products of the MHC³ and most cytotoxic T cells recognize antigen plus the class I MHC gene product⁴. There is still a question as to whether all suppressor T cells are MHC-restricted and which particular MHC gene product(s) they recognize⁵.

The T-cell antigen receptor is a heterodimer consisting of two disulphide-bridged polypeptide chains, α and β ⁶⁻⁸. Several cDNA clones encoded by both the human and mouse β -chain gene families have been isolated⁹⁻¹¹ and contain regions similar to immunoglobulin variable (V) and joining (J) gene segments and constant-region (C) genes^{9,11,12}. The genes encoding the β -polypeptide are located on chromosome 6 of the mouse^{13,14} and analysis of the germ-line β -chain gene organization provides further evidence for similarity with immunoglobulin gene families¹⁵⁻²¹. The germ-line DNA contains separate V_β , diversity (D_β) and J_β gene segments that rearrange in T lymphocytes to form complete V_β genes. These rearrangements apparently are mediated by recognition signals similar to those of immunoglobulin gene segments located just 3' of V_β gene segments, 5' of J_β gene segments and on both sides of the D_β gene segments.

Unique features of the β -chain gene family include the C_β gene arrangement; the closely linked $C_\beta 1$ and $C_\beta 2$ are each preceded by a cluster of six functional J_β gene segments^{15,17,18} and at least one functional D_β gene segment (Fig. 1)¹⁹⁻²¹. Thus, V_β gene segments may rearrange directly to either C_β gene. Here, we characterize β -chain DNA rearrangements and transcripts in several T lymphocytes and lymphoid tissues and have begun to elucidate the mechanisms of rearrangement in the β -chain gene family. We demonstrated that some suppressor cells do not use the β -chain genes and therefore may have antigen receptors encoded by a different gene family and we have tested the possible functional significance of $C_\beta 1$ and $C_\beta 2$ gene expression.

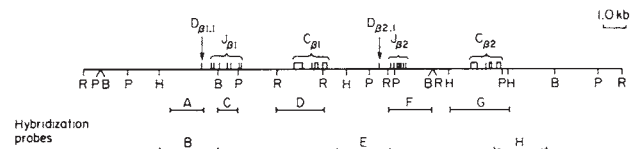


Fig. 1 Germ-line T-cell antigen receptor β -chain genes showing ~ 29 kb DNA from the β -chain gene locus. Exons are depicted by vertical lines or boxes above the thin horizontal line. The two known D_β gene segments, the two clusters of seven J_β gene segments and the four exons encoding each of the two C_β genes are indicated¹⁵⁻²¹. Restriction enzyme cleavage sites are represented by vertical lines below the horizontal line. Abbreviations: B, *Bam*HI; H, *Hind*III; P, *Pvu*II; R, *Eco*RI. The various features are drawn approximately to scale. Hybridization probes were purified restriction fragments derived either from the germ-line cosmid clone 2.3W7 or from subclones made from this germ-line β -chain gene cosmid clone or purified restriction fragments from a $C_\beta 1$ cDNA clone. They include the following: Probe A detects rearrangement of $D_\beta 1-1$ or its surrounding sequences. It is a 1.8-kb *Pst*I fragment previously subcloned into M13mp8. Probe B hybridizes to $D_\beta 1-1$, $J_\beta 1-1$ and $J_\beta 1-2$ and is a 3.2-kb *Hind*III/*Bam*HI fragment previously subcloned into pUC8. Probe C hybridizes to the $J_\beta 1-3$, $J_\beta 1-4$ and $J_\beta 1-5$ gene segments. It is a *Bam*/*Pvu*II fragment derived from a 6.2-kb *Bam*/*Hind*III fragment subcloned into pUC8. Probe D, which includes the $C_\beta 1$ gene, is a 2.5-kb *Eco*RI fragment derived from the same 6.2-kb *Bam*/*Hind*III subclone described above. Probe E hybridizes to $D_\beta 2-1$ and is a 2.4-kb *Hind*III/*Eco*RI fragment previously subcloned into M13mp18. Probe F is a 2.3-kb *Eco*RI fragment derived from a 4.5 kb *Bam*/*Hind*III fragment subcloned into pUC8 and hybridizing to the entire $J_\beta 2$ gene segment cluster. Probe G, which includes the $C_\beta 2$ gene, is a 3-kb *Hind*III fragment subcloned into pUC8. Probe H hybridizes to the 3'-nontranslated sequences of the $C_\beta 2$ transcript. It is a 2.5-kb *Sac*I fragment purified from a digest of the cosmid clone. Lastly, another $C_\beta 1$ probe, the 700-bp *Pst*I fragment of the RBL5-17 cDNA clone which contains most of the C region and 3'-untranslated sequence of $C_\beta 1$ (ref. 13), is not shown in the figure. Because of their close sequence similarity, the $C_\beta 1$ cDNA probe, probe D or probe G could generally be used to detect both the $C_\beta 1$ and $C_\beta 2$ genes.

Multiple β -chain gene rearrangements

The organization of the mouse β -chain gene family near the two C_β genes is shown in Fig. 1. DNA from the cells described in Table 1 was digested with several restriction enzymes and Southern blots of the digested DNA then hybridized with several

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Table 1 β -chain gene segment rearrangements in T lymphocytes

Cell	Source	Specificity	Interpretation		Comments
Helper T cells					
L2	Fitch	MIs ^a	1r/1d	2r	<i>C_β</i> 1 deleted, possible <i>D_β</i> 1-1- <i>J_β</i> 2 joining <i>D_β</i> 1-1- <i>J_β</i> 1-1 and two <i>D_β</i> 2-1- <i>J_β</i> 2 partial rearrangements
LP.83.A	Delovitch	I-A ^k	2r/1g	2r/1g	
HT-1	Watson	Sheep red blood cells/I-A ^b	1r/1?	1r/1g	
3H-25	Sercarz	Lysozyme/I-A ^b	1r/1d	2r	<i>D_β</i> 2- <i>J_β</i> 2 partial rearrangement
1.2	Sercarz	Lysozyme/I-A ^b	1r/1d	1g/1d	One chromosome may have been lost
1.3	Sercarz	Lysozyme/I-A ^b	2r	1r/1?	One or two <i>D_β</i> 2- <i>J_β</i> 2 partial rearrangements
1.4	Sercarz	Lysozyme/I-A ^b	2r	3r	<i>D_β</i> 2- <i>J_β</i> 2-partial rearrangement
1.7	Sercarz	Lysozyme/I-A ^b	1r/1d	2r	<i>D_β</i> 2- <i>J_β</i> 2 partial rearrangement
AT11.1	M. Pierres	GAT/I-A ^k	1r/1?	1r/1g	
B8/C3X/B ⁻	Shevach	Insulin/I-A ^d	2d	4r	<i>C_β</i> 1 deleted also
11.5	Hansburg	Cytochrome <i>c</i> /I-E ^k	2r	2r	
15.4	Hansburg	Cytochrome <i>c</i> /I-E ^k	2r	2r	
1.9.2	Hansburg	Cytochrome <i>c</i> /I-E ^k	1r/1d	2r	
Cytotoxic T cells					
4B3	R. Miller	H-2 ^d	2d	ND	<i>C_β</i> 1 deleted, <i>C_β</i> 2 germ line
4A2	R. Miller	H-2 ^d	2?	ND	Deleted up to <i>J_β</i> 1-2, <i>C_β</i> 1 germ line
CTLL-2	Dennert	None	2r	1r/1g	
L-137	Delovitch	I-A ^k	2r	2r	<i>D_β</i> 2- <i>J_β</i> 2 partial rearrangement
L-36	Delovitch	I-A ^k	1r/1?	1r/1?	
L-73	Delovitch	I-A ^k	1r/1?	n.d.	
14-13	Braciale	Influenza/K ^d	2d	1r/1d	One chromosome may have been lost
14-7	Braciale	Influenza/K ^d	2?	2r	Germ line from <i>J_β</i> 1-3 to <i>J_β</i> 1-7
14-1	Braciale	Influenza/K ^d	3r	1r/1g	
11-1	Braciale	Influenza/K ^d	1r/1d	2r	
Tumour					
S49	Cohn	Mineral oil	1r/1d	2r	<i>D_β</i> 1-1- <i>J_β</i> 1 and <i>D_β</i> 2-1- <i>J_β</i> 2 partial rearrangement
BAL3	Asofsky	Ethylnitrosamine	2r	2r	Two <i>D_β</i> 1-1- <i>J_β</i> 1-1 and a <i>D_β</i> 2-1- <i>J_β</i> 2 partial rearrangement
WEHI 22	Warner	Spontaneous	2d	2r	<i>C_β</i> 1 deleted also
KKT-2	Weissman	Spontaneous	1r/1d	2r	<i>D_β</i> 1-1- <i>J_β</i> 1 and <i>D_β</i> 2-1- <i>J_β</i> 2 partial rearrangement
SL3	E. Hayes	Spontaneous	2r/1g	1r/1?	Two copies of chromosome 6
SL15	E. Hayes	Spontaneous	ND	1r/1g	
SL17	E. Hayes	Spontaneous	ND	1r/1d	
BW5147	Hyman	Spontaneous	2d	2r	<i>C_β</i> 1 deleted also
EL4	Boyse	Carcinogen	1r/1d	1r/1g	<i>D_β</i> 1-1- <i>J_β</i> 1 partial rearrangement
RL17	Kaplan	Radiation	2d	2r	<i>C_β</i> 1 deleted also
VL3	Kaplan	Radiation	2d	2r	<i>C_β</i> 1 deleted also
AQR	Meruelo	Viral	2d	3r	<i>C_β</i> 1 deleted also
A13	Cheseborough	Viral	1r/1d	1r/1g	<i>D_β</i> 1-1- <i>J_β</i> 1 partial rearrangement
UNC-1	Haughton	?	1r/1d	2r	<i>D_β</i> 1-1- <i>J_β</i> 1 partial rearrangement
Suppressor T-cell hybridomas					
763B3.7	Kapp/Pierce	GAT, GA	1r/1d	2r	<i>D_β</i> 1-1- <i>J_β</i> 2 partial rearrangement
258C4.4	Kapp/Pierce	GAT, GA	2d	2d	All <i>D_β</i> - <i>J_β</i> - <i>C_β</i> deleted
372B3.5	Kapp/Pierce	GAT, GT	2d	2d	All <i>D_β</i> - <i>J_β</i> - <i>C_β</i> deleted
372.D6.5	Kapp/Pierce	GAT, GT	2d	2d	All <i>D_β</i> - <i>J_β</i> - <i>C_β</i> deleted
258C4.5	Kapp/Pierce	None	2d	2d	All <i>D_β</i> - <i>J_β</i> - <i>C_β</i> deleted

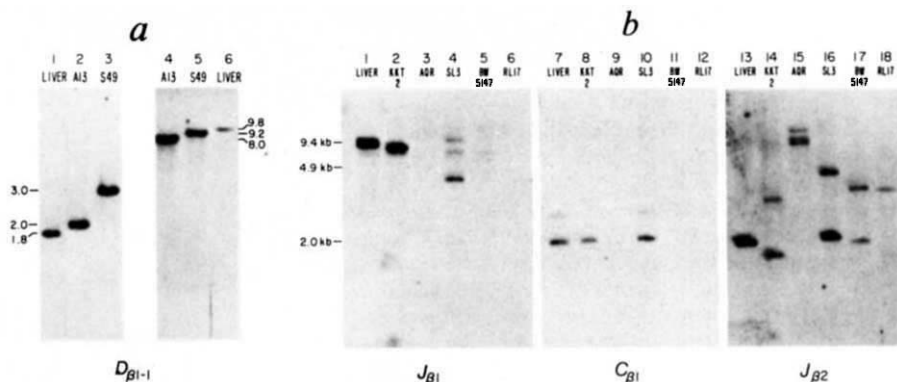
T lymphocytes are grouped by category. The antigen specificity, if any, of the tumours is uncertain and the method of induction is listed instead. Cytotoxic cell CTLL-2 and suppressor hybridoma 258C4.5 have lost the ability to respond to antigen. r, Rearranged; d, deleted; g, germ line, ?, unknown. In several cases, a rearrangement could not be distinguished from a deletion with the probes used. ND, not done. Five of the helper cells are hybridomas where rearrangements derived from the BW5147 T-lymphoma fusion partner were not included. Some of the hybridization results important for the interpretations not presented in Figs 2 and 3 will be published elsewhere (ref. 39 and J. Kobori, N. Shastri and L.H., manuscript in preparation). All T cells were provided by the laboratories listed under source, except for helper T-cell hybridomas AT11.1 and B8/C3X/B⁻ provided by the laboratory of Dr T. Delovitch and tumours which were provided by the laboratory of Dr I. Weissman, or by the laboratory of Dr E. Hayes.

probes, allowing us to determine the number and location of the DNA rearrangements, which in many instances could be localized to one or two particular J_{β} gene segments (Figs 2, 3).

All the murine T helper, killer and lymphomas tested contained rearranged β -chain genes (Table 1, Figs 2, 3). We hybridized Southern blots of DNA isolated from 35 T cells of various types with $J_{\beta}1$ gene segment probes. Eight have deleted the $J_{\beta}1$ gene segment clusters from both chromosome 6 homologues, 11 have one deleted and one rearranged $J_{\beta}1$ gene segment cluster, seven have two $J_{\beta}1$ rearrangements and three had three restriction fragments which hybridized with $J_{\beta}1$ probes. In six cells, one or both $J_{\beta}1$ gene segment clusters were not fully determined, usually because a rearrangement could not be distinguished from a deletion with the combinations of probes and

restriction enzymes used here. Therefore, of a total of 65 $J_{\beta}1$ gene segment clusters analysed, 36 rearranged, 27 deleted and only 2 were in the germ-line configuration. Of 34 T cells tested for $J_{\beta}2$ gene segment rearrangement, none has deleted both $J_{\beta}2$ gene segment clusters, although three cells apparently have one rearrangement and one deletion, seven have one rearranged and one germ-line $J_{\beta}2$ gene segment cluster and 17 have two $J_{\beta}2$ rearrangements. As observed for the $J_{\beta}1$ hybridizations, four T cells have more than two fragments hybridizing with $J_{\beta}2$ probes whereas three other T cells have one homologue not analysed completely. From a total of 70 $J_{\beta}2$ gene segment clusters, 58 are rearranged; only nine are in the germ-line configuration and *tgaaogteghree* are deleted. Thus, only 3% of the $J_{\beta}1$ gene segment clusters and 13% of the $J_{\beta}2$ gene segment clusters were

Fig. 2 β -chain gene rearrangements in T-cell tumours. Conditions used for the Southern blots have been described previously⁶³. **a**, Hybridization of the $D_{\beta 1-1}$ probe (probe A in Fig. 1) to *Pst*I-digested DNA (lanes 1–3) and to *Eco*RI-digested DNA (lanes 4–6) from the A13 and S49 lymphomas and from mouse liver. The approximate sizes of the hybridizing restriction fragments are indicated. **b**, Hybridization of β -chain gene probes to *Eco*RI-digested DNA from five T-cell tumours. The probes used were: $J_{\beta 1}$ (probe B), lanes 1–6; the $C_{\beta 1}$ probe from the RBL-5 cDNA clone, lanes 7–12; $J_{\beta 2}$ (probe F), lanes 13–18. The same filter was hybridized sequentially with each of the three probes. The migration distances of some DNA restriction fragments of known length (kb) are indicated next to lane 1 in **a**. The faint hybridization of the $J_{\beta 1}$ probe to BW5147 DNA in lane 5 was not reproducible. Hybridization of the $C_{\beta 1}$ probe to $C_{\beta 2}$ sequences was visible only following longer exposures. RL17 shows one band when digested with *Eco*RI and hybridized with a $J_{\beta 2}$ probe (lane 18), but two $J_{\beta 2}$ bands when digested with other restriction enzymes (Table 1).



in the germ-line configuration. With the possible exception of one T-helper cell line (LP.83.A), no single chromosome 6 contains both the $J_{\beta 1}$ and $J_{\beta 2}$ gene segments in the germ-line configuration. A similar lack of germ-line $J_{\beta 1}$ and $J_{\beta 2}$ gene segments was observed when DNA from heterogeneous populations of normal T cells was hybridized with J_{β} gene segment probes (R.H. and M.K., manuscript in preparation), suggesting that the multiple rearrangements detected in T-cell lymphomas and functional cell lines are not an artefact of long-term cell culture or cell transformation. Considering separately the DNA joining events in the different categories of T cells, we find that the rearrangement patterns in helper and cytotoxic T lymphocytes are quite similar, whereas the tumours delete the $J_{\beta 1}$ gene segment clusters and $C_{\beta 1}$ genes more frequently than the functional cells. The biological significance of this observation is unclear.

In addition to V gene formation, immunoglobulin genes show DNA rearrangements associated with the switch in expression of heavy-chain class and with the translocation of proto-oncogenes. The heavy-chain class switch of immunoglobulin genes deletes the C_{μ} gene and joins the intron 5' to this gene with the intron 5' to the C_{H1} gene to be expressed²². The analogous rearrangement in the β -chain gene family would delete the $C_{\beta 1}$ gene and join the intron between $J_{\beta 1}$ and $C_{\beta 1}$ to the intron between the $J_{\beta 2}$ and the $C_{\beta 2}$ gene. In none of the T cells examined is there evidence for such a switch rearrangement, although this type of event cannot be excluded completely for several cells with complex β -chain gene hybridization patterns.

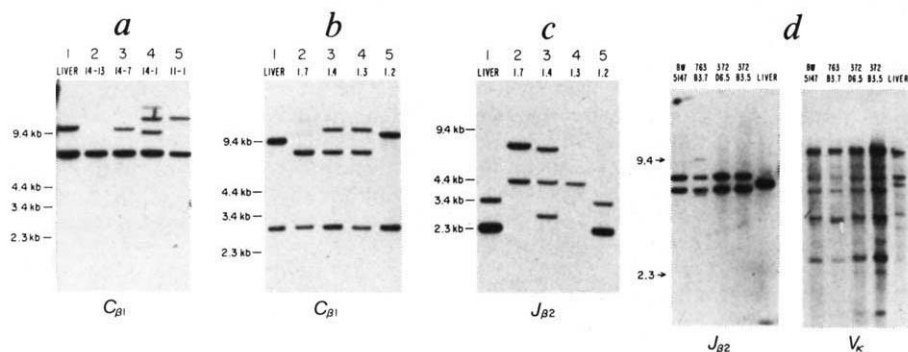
The apparent lack of class switching in the β -chain gene family is expected because, unlike the immunoglobulin heavy-chain gene family, each C_{β} gene has its own cluster of J_{β} gene segments to which V_{β} and D_{β} gene segments can be joined directly.

Rearrangement of proto-oncogenes to the immunoglobulin loci have been characterized in many B-cell tumours²³. Translocations involving chromosome 6, however, are not common in mouse T lymphomas²⁴ and, using a variety of probes, oncogene rearrangements have not yet been detected in the 14 T lymphomas analysed (R. K. Barth, personal communication). Therefore, we conclude that proto-oncogene rearrangements near C_{β} genes occur rarely in mouse T-cell tumours and that most of the rearrangements result from the normal process of V region gene formation.

Partial β -chain gene rearrangements

Expression of the immunoglobulin heavy and light chains shows allelic exclusion; that is, in any given B lymphocyte only one of the two homologues encoding the light- and heavy-chain gene families can encode a functional polypeptide which is part of a membrane-bound or secreted immunoglobulin molecule. One allele may be silent because it has not rearranged²⁵. In many normal B lymphocytes, however, the silent allele is rearranged^{26,27}, transcribed, and the resulting RNA may even be translated^{28–30}, although this second rearrangement is always nonproductive because it does not encode part of a functional immunoglobulin^{28–31}. Nonproductive rearrangements occur by several mechanisms, such as joining of gene segments out of

Fig. 3 β -chain gene rearrangements in helper, killer and suppressor T lymphocytes. The migration distances of some M_r markers are indicated. **a**, β -chain gene rearrangements in cloned cytotoxic T-lymphocyte lines specific for influenza A/JAP/305/57. All DNAs were digested with *Bam*HI and hybridized with the $C_{\beta 1}$ probe (probe D). The 10.8-kb M_r band observed in germ-line (liver) DNA contains the $C_{\beta 1}$ gene, whereas the 6.0-kb band contains the $C_{\beta 2}$ gene. Because there is a *Bam*HI site in the $J_{\beta 2}$ - $C_{\beta 2}$ intron, rearrangement of $C_{\beta 2}$ is not detected following *Bam*HI digestion. **b**, **c**, β -chain gene rearrangements in cloned helper T-lymphocyte lines from mice immunized with the cyanogen bromide fragment L2 of hen egg-white lysozyme. **b**, DNAs were digested with *Hind*III and hybridized with the $C_{\beta 1}$ probe (probe D). The 9.4-kb band in liver DNA contains the germ-line $C_{\beta 1}$ gene whereas the 3.0-kb band contains $C_{\beta 2}$ gene. Rearrangement of $C_{\beta 2}$ is not detected following *Hind*III digestion. **c**, DNAs were digested with *Eco*RI and hybridized with a $J_{\beta 2}$ probe which is larger than probe F and hybridizes to the *Eco*RI fragment corresponding to probe F and to the *Eco*RI fragment 5' to it, encompassing probe E. **d**, Gene deletions and rearrangements in suppressor T-cell hybridomas. Lanes 1–5, hybridization of the $J_{\beta 2}$ probe (probe F) to *Pvu*II-digested DNA from three suppressor hybridomas, BW5147, and from mouse liver DNA; lanes 6–10, hybridization of the $p_{\kappa}(11)$ probe to *Eco*RI-digested T-suppressor hybridoma, BW5147 lymphoma and mouse liver DNA.



the proper translational reading frame³²⁻³⁴ and incomplete or D_H - J_H rearrangement in the absence of V_H gene segment rearrangement³⁴⁻³⁶. Allelic exclusion has not been demonstrated for the T-cell antigen receptor and some 'dual-receptor' models for T-cell antigen recognition predict that more than one type of receptor from the same gene family is expressed on the surface of a T lymphocyte³⁷.

If allelic exclusion is a mechanism of β -chain expression, then most observed DNA rearrangements in T lymphocytes must be nonproductive, such as the observed partial rearrangement of D_β to J_β gene segments (Table 1). Because they do not include V_β gene segments, these rearrangements are nonproductive for antigen receptor biosynthesis, although as described for immunoglobulins³⁸, they sometimes are transcribed and possibly could give rise to functional D_β - J_β - C_β polypeptides^{20,21}. Partial rearrangements of the D_β 1-1 gene segment can be detected by hybridizing Southern blots of T-cell DNA with probe A (Fig. 1) which contains only the D_β 1-1 gene segment, its flanking sequences and no other known coding sequences. This D_β gene segment is only 12 nucleotides long and V_β - D_β - J_β rearrangements deleting both its 5'- and 3'-flanking sequence would leave too little homologous sequence for effective hybridization. Therefore, probe A will detect partial (V_β - D_β or D_β - J_β) but not complete (V_β - D_β - J_β) rearrangements. In several T cells, these D_β and J_β gene segment probes hybridized to an identically-sized fragment slightly smaller than the germ-line fragment (Fig. 2a, lanes 4-6 and data not shown), indicating a small deletion joining the D_β 1-1 to a J_β 1 gene segment.

To support this conclusion, we digested DNA from the same T cells with *Pst*I, which cuts once in the 647-base pair (bp) intron between the D_β 1-1 and J_β 1 gene segments²⁰; using probe A, we observed a hybridizing fragment larger than the germ-line fragment (Fig. 2a, lanes 1-3). The loss of this *Pst*I site confirms the deletion between D_β 1-1 and J_β 1-1; eight similar deletions were observed in seven T lymphocytes. Similar evidence for joining of D_β 2-1 to J_β 2 gene segments has been presented previously²⁰ and we describe here at least 10 examples of this incomplete rearrangement (Table 1). Finally, a D_β 1-1 to J_β 2 rearrangement is consistent with the Southern blot patterns from two other T cells (Table 1) and the restriction map of a genomic clone isolated from a third cell line³⁹. All the characterized partial rearrangements of D_β gene segments delete the 3'-flanking sequence and join a D_β to a J_β gene segment. Therefore, any D_β gene segment seems to be capable of joining to any J_β gene segment located 3' to it and there is no evidence for partial V - D rearrangements, a situation similar to immunoglobulin heavy chains⁴⁰. By further analogy with the immunoglobulin heavy-chain genes⁴⁰, we predict that the required initial event in the assembly of a complete V_β gene will be a D_β - J_β gene segment joining.

Two T lymphocytes, clones 14-13 (an influenza-specific cytotoxic cell) and 1.2 (a lysozyme-specific helper T cell), contain a single J_β gene segment rearrangement (Fig. 3, Table 1). Helper cell line LP.83.A has four J_β rearrangements, three of which result from partial D_β - J_β joining (Table 1). Therefore, only one productive β -chain gene rearrangement is required for these T lymphocytes to recognize and respond to antigen, which suggests that allelic exclusion of β -chain expression operates in T cells. However, formal proof of allelic exclusion would require the demonstration that all T lymphocytes have only one productive β -chain gene rearrangement.

Mechanism of β -chain gene rearrangement

Immunoglobulin gene-segment joining might occur by looping out and excising the DNA between the joined gene segments (refs 41, 42 and Fig. 4a). If we apply this model to the β -chain genes, any complete rearrangement involving a J_β 2 gene segment as well as any partial rearrangement (except those involving the D_β 2-1 gene segment) should delete both the J_β 1 gene segment cluster and the C_β 1 gene. Many of the β -chain gene rearrangements are consistent with this mechanism, including the partial D_β - J_β joining described above. In addition, 8 of 35 T cells

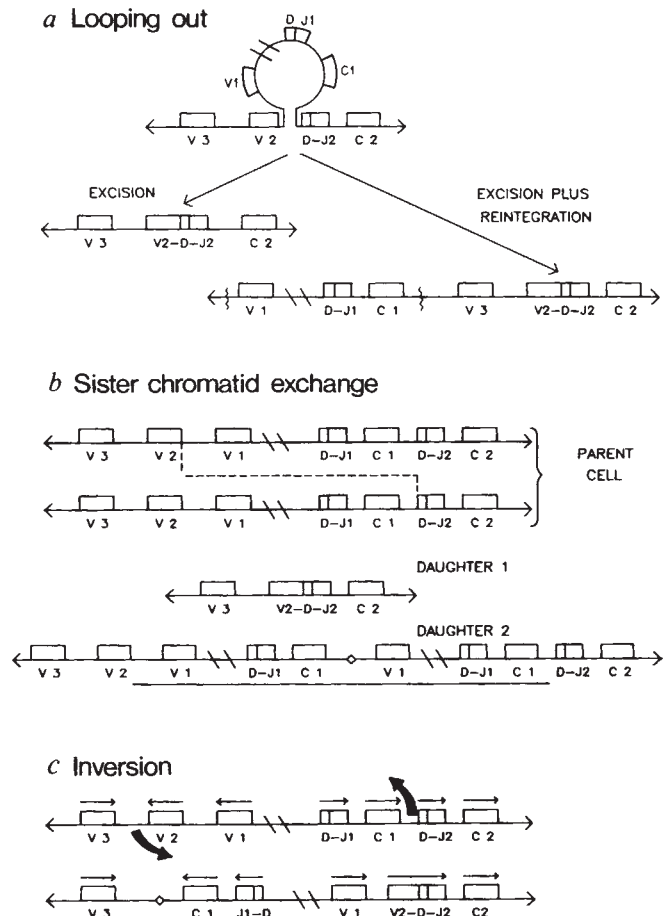


Fig. 4 Four models for T-cell antigen receptor and immunoglobulin gene rearrangement. Rearrangements are shown schematically in a gene family with organizational features similar to the β -chain gene family. V , D and J gene segments and C genes, represented by boxes above the horizontal line, are not drawn to scale. The double diagonal line shows that V gene segments have not been physically linked to D or J gene segments in any of these gene families. For clarity, only rearrangement of a hypothetical V_2 gene segment to an already-joined D - J_2 gene segment is illustrated, although the same models apply to D - J gene segment rearrangements. **a**, A stem and loop is formed by base-paired DNA joining recognition sequences^{41,42}. The broken vertical lines indicate the boundaries of the reintegrated sequences. Although reintegration to the same chromosome is indicated, these sequences may reintegrate to any chromosome. **b**, Unequal sister chromatid exchange. The right-hand brackets represent the connection of the sister chromatids at the centromere. The sequences duplicated on one of the two segregated chromosomes are underlined. The diamond on the horizontal line between C_1 and V_1 denotes a recombination breakpoint. **c**, Inversion. The horizontal arrows indicate the transcriptional orientation of the various gene segments and C genes.

analysed do not hybridize with J_β 1 (Fig. 2b, lanes 3, 5-6) and C_β 1 (Fig. 2b, lanes 9, 11-12) probes and therefore have presumably deleted these genes from both chromosomes. Each of these T lymphocytes also has several rearrangements in the J_β 2 gene segment cluster (Fig. 2b, lanes 15, 17-18), suggesting that a V_β - D_β - J_β 2 or D_β - J_β 2 joining event has occurred on both homologues via deletion of the intervening DNA.

Because B lymphocytes often retain sequences located in the germ line between rearranged V_k and J_k gene segments and therefore should have been deleted according to the model described above⁴³⁻⁴⁸, three other mechanisms have been proposed. Rearrangement could occur by either unequal sister chromatid exchange (Fig. 4b) or by an unequal mitotic recombination between chromosomal homologues⁴⁴⁻⁴⁶. A mitotic crossover in a heterozygous animal would lead to a recombinant

antigen receptor polypeptide with a V gene segment encoded by one parent and J and C encoded by the other. There is only indirect evidence for this type of recombinant immunoglobulin molecule, however, and such molecules are not seen often in F_1 heterozygotes⁴⁹. Therefore, unequal sister chromatid exchange is the most probable of these two mechanisms. A further model involving an inversion mechanism could also join the various gene segments (Fig. 4c)^{47,50,51}. Because V gene segments have not been linked physically with J gene segments in any of the immunoglobulin gene families, it is not known whether all of these gene segments are in the same transcriptional orientation. Finally, it is possible that an excision does occur during gene segment rearrangement, but the excised sequences then may reintegrate elsewhere (Fig. 4a)^{43,44}. We need not consider this to be an alternative mechanism, but simply one possible outcome following an excision event.

Similar to κ -chain genes in B cells, we have found that the β -chain gene rearrangements in at least 13 of the 37 T cells analysed cannot easily be explained by the looping-out and excision mechanism and are therefore better explained by one of the other mechanisms illustrated in Fig. 4. These T cells have either retained sequences located in the germ line between two joined gene segments, or have more restriction fragments hybridizing with β -chain gene probes than expected in cells diploid for chromosome 6. For example, a large fraction of the T cells have one or several rearranged $J_{\beta 1}$ gene segment clusters as well as several $J_{\beta 2}$ gene segment rearrangements. In nine of these cells, the number of $J_{\beta 1}$ gene segment rearrangements equals or exceeds the number of $J_{\beta 2}$ gene segment rearrangements which are not due to partial ($D_{\beta 2-1}$ - $J_{\beta 2}$) joining. In these nine cells, if the $J_{\beta 2}$ gene segments had been rearranged by deletion, at least one of the observed rearranged $J_{\beta 1}$ gene segments should not have been present in the genome (see Fig. 3). Clone 1.4, a hen egg lysozyme-specific T-helper cell line, has two rearranged and no germ-line $J_{\beta 1}$ gene segment clusters (Fig. 3b, lane 3). Using a $J_{\beta 2}$ probe, clone 1.4 has three rearrangements and no germ-line restriction fragments (Fig. 3c, lane 3). Although one of these $J_{\beta 2}$ gene segment rearrangements is a small deletion probably resulting from a $D_{\beta 2-1}$ joining (data not shown), the other two are not and therefore should have deleted the two $J_{\beta 1}$ gene segment clusters.

The presence in clone 1.4 of three rearranged restriction fragments containing $J_{\beta 2}$ gene segments and a similar hybridization pattern in five other T lymphocytes, may also indicate that some of these rearrangements were not caused by simple excisions. Three cells have three restriction fragments containing $J_{\beta 1}$ gene segment sequences; four cells, including one of the cells showing three $J_{\beta 1}$ rearrangements, have three or four restriction fragments hybridizing with $J_{\beta 2}$ gene segment probes (Table 1 and Fig. 2b, lanes 4, 15; Fig. 3c, lane 3). If $D_{\beta 2}$ - $J_{\beta 2}$ joining occurred by one of the three alternatives to a simple excision (Fig. 4), a single chromosomal homologue containing two restriction fragments which hybridize with a J_{β} probe and consequently a diploid T lymphocyte with three or four such fragments could result. Although these patterns would also occur if a T lymphocyte became triploid for chromosome 6 and then underwent a subsequent J_{β} gene segment rearrangement, this is unlikely in such a large fraction (6 of 37) of the T cells analysed. In addition, cytogenetic analysis of the SL3 lymphoma, which has three restriction fragments detected by a $J_{\beta 1}$ probe, demonstrated that this cell has only two copies of chromosome 6 (J. Sawyer and J. Hosier, personal communication), although a small extra translocated fragment of chromosome 6 might be undetected by this analysis.

Models for β -chain gene rearrangement requiring sister chromatid exchange or mitotic recombination can be distinguished from inversions, as well as reintegration of excised sequences, because these latter events do not change the number of J_{β} gene segments and C_{β} genes in the genome of a cell; they merely change the genomic context of some of these sequences (Fig. 4). For example, an inversion in J_{β} gene segment clusters would create an extra fragment hybridizing with J_{β} probes by

removing some of the J_{β} gene segments from the vicinity of a C_{β} gene. By contrast, unequal sister chromatid exchange involving a $J_{\beta 2}$ gene segment would result in a chromosome containing duplicated $J_{\beta 1}$ gene segments and $C_{\beta 1}$ genes (Fig. 4b) and consequently could result in a cell having more than the diploid number of such sequences. To distinguish between the models, Southern blots of DNA from two T cells containing three $J_{\beta 1}$ restriction fragments were hybridized with a $C_{\beta 1}$ probe after digestion with restriction enzymes which do not cut between the $J_{\beta 1}$ gene segments and the $C_{\beta 1}$ gene. Clone 14-1 (Fig. 3a, lane 4) contained three rearranged C_{β} gene restriction fragments, whereas SL3 had only two hybridizing fragments (data not shown), demonstrating that in clone 14-1 but not in SL3, all three fragments hybridizing with $J_{\beta 1}$ gene segment probes are adjacent to, but not separate from, the $C_{\beta 1}$ gene. Therefore, the presence of three copies of the $C_{\beta 1}$ gene in clone 14-1 is consistent with the mechanism of unequal sister chromatid exchange, whereas the rearrangements in SL3 may be better explained by inversions or by reintegration of excised sequences. It seems that several mechanisms, perhaps all those in Fig. 4, may operate to rearrange β -chain gene segments.

β -chain gene deletion

Five suppressor T-cell hybridomas were examined for β -chain gene rearrangement. The hybrids were constructed by fusing lymphocytes immune to the synthetic terpolymer of L-glutamic acid⁶⁰-L-alanine³⁰-L-tyrosine¹⁰ (GAT) or closely related antigens with the AKR strain T-lymphoma BW5147^{52,53}. This lymphoma has no J_{β} gene segments in the germ-line configuration but has deleted both of its $J_{\beta 1}$ gene segment clusters and $C_{\beta 1}$ genes and has two $J_{\beta 2}$ gene segment rearrangements (Fig. 2b, lanes 5, 11, 17). As it is highly improbable that two independently derived T lymphocytes will have identical rearrangements, any germ-line or rearranged J_{β} gene segments from the suppressor T-cell fusion partner in the hybridomas should be distinguishable from the rearranged fragments derived from BW5147 lymphoma DNA. Using a series of β -chain gene probes, we observed that DNA from four of the suppressor T hybridomas, when digested separately with several restriction enzymes, have hybridization patterns identical to BW5147 (Fig. 3d and data not shown). Therefore, the entire set of D_{β} - J_{β} - C_{β} genes shown in Fig. 1 have been deleted from both chromosome 6 homologues derived from the suppressor T-cell fusion partner in these hybridomas. We have shown recently that the deletion in two of these hybridomas that were further tested includes V_{β} genes as well (M.K. and R. Barth, unpublished observations).

The J_{β} clusters and C_{β} genes may have been deleted during V_{β} rearrangement to suppressor T-cell J_{β} segments and C_{β} genes, located downstream of $C_{\beta 2}$. Because the murine κ locus is linked to the β -chain gene locus, we hybridized Southern blots of the T-suppressor cell DNA with the κ probe, $p_{\kappa}(11)$, a cDNA clone (provided by Dr R. Riblet) encoding the κ constant and part of a V region expressed by the MPC-11 myeloma⁵⁴. On Southern blots, $p_{\kappa}(11)$ detects polymorphic restriction fragments which distinguish the V_{κ} gene segments of the AKR strain of the BW5147 hybridoma fusion partner from the V_{κ} gene segments of most other mouse strains. In four of five suppressor hybridomas, the pattern obtained with the κ probe was identical to BW5147 (Fig. 3d), indicating that the analysed V_{κ} as well as the β -chain genes of the normal parent had been deleted. Although the precise distance between the κ - and β -chain gene loci is unknown, it is unlikely that any V_{κ} gene segments are located either in the middle of the V_{β} gene segments or between these segments and a putative suppressor-cell C_{β} gene. We therefore conclude that the deletion in these cells extends beyond the β -chain gene locus and is not caused by rearrangement of a V_{β} gene segment to a downstream suppressor C_{β} gene. The observed deletions in suppressor hybridomas which include at least part of the β - and κ -chain gene loci could be losses of the entire chromosome, or they could be analogous to the deletions of the κ -chain genes in most human B cells which synthesize λ light chains⁵⁵.

Human T-suppressor cell lines^{56,57} and some mouse T-suppressor hybridomas (Fig. 3d) do have β -chain gene rearrangements, although many of these rearrangements may be nonproductive. At least one human T-suppressor cell line, however, expresses a heterodimeric protein similar to the T-cell antigen receptors found on helper and cytotoxic T lymphocytes⁵⁸. The deletion of the β -chain gene locus in the suppressor hybridomas can therefore be explained in two ways. First, it is possible that some antigen-specific suppressor T cells do not express β -chain genes and therefore part of their receptor must be encoded by another, as yet undefined, gene family. T-suppressor cells may therefore fall into two or more groups on the basis of antigen receptor expression. Second, there may be some technical problem occurring repeatedly in different hybridomas. For example, T-cell hybridomas often lose chromosomes; the functional activity in the cells tested might therefore reside in the small subpopulation that retains the β -chain gene locus and was not detected in these experiments, implying that the BW5147-derived homologues are retained preferentially in these hybrid cells.

β -chain gene transcription

The murine $C_{\beta}1$ and $C_{\beta}2$ genes share a high level of similarity in their coding sequences (96%)^{17,18}, but the presence of two closely linked C_{β} genes in the murine and human genomes (B. Toyonaga, personal communication) suggests that they have some functional significance. We therefore used probes derived from the 3'-nontranslated portions of the $C_{\beta}1$ and $C_{\beta}2$ genes, identical for only 103 of 217 nucleotides¹⁸, which can distinguish between the two C_{β} transcripts (Table 2). There are two predominant C_{β} transcripts in T lymphocytes and lymphoid tissues: a 1.3-kb RNA containing a complete V region sequence^{9,11,12,15,21} and a 1.0-kb RNA derived from a partially rearranged (D_{β} - J_{β}) gene^{20,21}. All of the 14 functional helper and cytotoxic lymphocytes and 8 of 9 T lymphomas contain a 1.3-kilobase (kb) C_{β} transcript (Table 2 and our unpublished data). Occasionally, we found transcripts in some cells which do not fall into either size class (Table 2). The presence of a 1.3-kb transcript in all these cell lines suggests that they use β -chains as part of their antigen receptor.

The $C_{\beta}2$ gene is expressed detectably in many more of these cell lines (Table 2) as well as thymus, spleen and lymph node (data not shown), than is the $C_{\beta}1$ gene. Two C57BL/6 cytotoxic T-cell clones specific for H-2D^d haplotype MHC-gene products had different C_{β} transcription patterns; clone 4B3 contained only a 1.3-kb $C_{\beta}2$ RNA whereas clone 4A2 had both 1.3-kb $C_{\beta}1$ and 1.0-kb $C_{\beta}2$ transcripts (Table 2). Because the 1.0-kb C_{β} transcripts are derived from a partially rearranged (D_{β} - J_{β}) gene, we assume that in clone 4A3 the 1.3-kb $C_{\beta}1$ RNA encodes the functional β -chain. Therefore, cytotoxic T lymphocytes specific for MHC-encoded alloantigens can use either C_{β} constant region. Most of the helper T cells tested synthesize a 1.3-kb $C_{\beta}2$ RNA, suggesting that both helper and cytotoxic T lymphocytes can use the same C_{β} gene. The previously published nucleotide sequences of a β -chain cDNA clone from a helper T-cell hybridoma¹⁵ and a β -chain cDNA from a cytotoxic T-cell line confirm this suggestion for $C_{\beta}2$ (ref. 11), although none of the helper T-cell lines analysed in Table 2 synthesized only a 1.3-kb $C_{\beta}1$ RNA.

A single rearrangement involving the $J_{\beta}1$ gene segments in T-helper cell clone LP.83.A is the only one that can possibly be productive, and a lysozyme-specific T helper uses the $C_{\beta}1$ gene in its β -chain³⁹. Therefore, T-helper cells, as well as cytotoxic T cells, can synthesize β -chains using the $C_{\beta}1$ gene. One T lymphoma and three helper T-cell lines synthesize both a $C_{\beta}1$ and a $C_{\beta}2$ RNA large enough to encode a functional β -chain polypeptide. This does not necessarily contradict the allelic exclusion hypothesis, because transcription could occur from a nonproductive V_{β} - D_{β} - J_{β} rearrangement.

The KKT-2 lymphoma is exceptional in that it makes very little or no 1.3-kb transcript. Of the three β -chain gene rearrangements in this cell, at least two are partial (D_{β} - J_{β}) rearrangements

Table 2 C_{β} transcripts in tumours and T-lymphocyte lines

	$C_{\beta}1$	$C_{\beta}2$
Helper T cells		
AT11.1	1.3	1.3
CB7-4G2	1.3	1.3
LP.72	—	1.3
B8/C3X/6.2	1.0	1.3
LP.11	—	1.3
LP.18.9	1.5	1.3
Cytotoxic T cells		
4A2	1.3	1.0
4B3	—	1.3
L-137	—	1.3
Tumours		
S49	1.0	1.3
KKT-2	1.0	1.0
BW5147	—	1.3
EL4	—	1.3
VL3	—	1.3
UNC-1	1.0	1.3
L691	1.3, 0.8	1.3, 1.0
SPRD	—	1.3
WEHI7	—	1.3

RNA was purified and Northern blots carried out as described previously^{61,62}. The $C_{\beta}1$ -specific probe is an *EcoRI*/*PstI* fragment from the RBL5-17 cDNA clone containing almost 200 bp of the 3'-nontranslated $C_{\beta}1$ sequence. The $C_{\beta}2$ -specific probe is probe H described in Fig. 1. A dash indicates that no hybridization was detected, although in some cases only small amounts of RNA were tested and thus is not proof of the absence of a transcript. T cells were provided by the sources indicated in Table 1. In addition, the helper T-cell lines and hybridomas not listed in Table 1 were provided by Dr T. Delovitch and the EL4, SPRD and WEHI7 tumours by Dr James Allison. CB7-4G2 and B8/C3X/6.2 are insulin-specific T-cell hybridomas made by P. Wassmer and L. Glimcher in the laboratory of Dr E. Shevach. LP7 is an I-E^k-specific helper T cell, LP.11 and LP.18.9 are I-A^k-specific helper T cells made by Drs L. Phillips and T. Delovitch.

and each of these two gives rise to a different 1.0-kb RNA (Table 2). If D_{β} - J_{β} rearrangements do occur initially in development, this tumour may therefore be frozen at a relatively early stage in T-cell ontogeny. The data in Tables 1 and 2 indicate that partial rearrangement may be necessary, but not sufficient, to generate a C_{β} RNA synthesized from the promoters 5' of D_{β} gene segments.

T-cell receptor and T-cell function

Helper, cytotoxic and suppressor T cells are distinguished by both the MHC-encoded molecules they recognize and the functions they carry out following activation by the antigen. The antigen receptor may not be involved in distinguishing different T-cell functional classes, thus the antigen receptors from a helper or a cytotoxic T cell could not be distinguished from sequence information alone. Alternatively, the receptor may be related intimately to the T-cell functional class, analogous to the different functions associated with the different immunoglobulin isotypes. This latter view is supported by evidence of specific helper and suppressor T-cell factors which are antigen-binding proteins secreted by antigen-specific lymphocytes carrying out the immune function of the secreting cell⁵⁹. No antigen-specific T-cell factor, however, has yet been purified completely and characterized biochemically.

The data presented here support both views of the relationship between the antigen receptor and T-cell function. For helper and killer T cells and T lymphomas, β -chain genes are always rearranged and a 1.3-kb C_{β} transcript is present in all the different mouse and human⁶⁰ T helpers and cytotoxic T cells tested, hence β -chain genes are analogous to κ light-chain genes. The κ -chain genes are expressed in nearly every mouse B cell, regardless of the maturity or location of that cell, unlike the individual heavy-chain genes which are only expressed on limited subsets of B lymphocytes. In addition, the data presented here and elsewhere^{11,15} demonstrate that the two highly similar

C_β genes are functionally equivalent: either one (or both) can be expressed by helpers as well as cytotoxic T lymphocytes. Expression of a particular C_β gene does not seem to be a critical determinant of helper versus cytotoxic function, neither does it seem to be important in distinguishing recognition of class I versus class II MHC-encoded molecules. It remains possible, however, that helpers and cytotoxic T lymphocytes use different sets of V_β gene segments or that the α -chains are different in these cell types.

By contrast, data derived from suppressor T-cell hybridomas suggest that expression of particular types of T-cell receptors is related to the T-cell functional class. These data are negative, however; a number of suppressor T-cell hybridomas delete rather than rearrange their β -chain genes and a firm proof of this hypothesis will require isolation of the genes encoding this putative T-suppressor antigen receptor.

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1. Golub, E. *Cell* **21**, 603-604 (1980).
2. Matzinger, P. & Zamoyska, R. *Nature* **297**, 628 (1982).
3. Thomas, D. W., Yamashita, U. & Shevach, E. M. *J. Immun.* **119**, 223-226 (1977).
4. Doherty, P. C. & Zinkernagel, R. M. *J. exp. Med.* **141**, 502-507 (1975).
5. Germain, R. N. & Benacerraf, B. *Scand. J. Immun.* **13**, 1-10 (1981).
6. Allison, J. P., McIntyre, B. W. & Bloch, D. J. *Immun.* **129**, 2293-2300 (1982).
7. Haskins, K., Kubo, R., White, J., Pigeon, M., Kappler, J. & Marrack, P. *J. exp. Med.* **157**, 1149-1169 (1983).
8. Meuer, S. C. *et al. J. exp. Med.* **157**, 705-719 (1983).
9. Yanagi, Y. *et al. Nature* **308**, 145-149 (1984).
10. Hedrick, S. M., Cohen, D. I., Nielsen, E. A. & Davis, M. M. *Nature* **308**, 149-153 (1984).
11. Saito, H. *et al. Nature* **309**, 757-762 (1984).
12. Hedrick, S. M., Nielsen, E. A., Kavaler, J., Cohen, D. I. & Davis, M. M. *Nature* **308**, 153-158 (1984).
13. Caccia, N. *et al. Cell* **37**, 1091-1099 (1984).
14. Lee, N. E. *et al. J. exp. Med.* **160**, 905-913 (1984).
15. Chien, Y.-H., Gascoigne, N. R. J., Kavaler, J., Lee, N. E. & Davis, M. M. *Nature* **309**, 322-326 (1984).
16. Siu, G. *et al. Cell* **37**, 393-401 (1984).
17. Malissen, M. *et al. Cell* **37**, 1101-1110 (1984).
18. Gascoigne, N. R. J., Chien, Y.-H., Becker, D. M., Kavaler, J. & Davis, M. M. *Nature* **310**, 387-391 (1984).
19. Kavaler, J., Davis, M. M. & Chien, Y.-H. *Nature* **310**, 421-423 (1984).
20. Siu, G. *et al. Nature* **311**, 344-349 (1984).
21. Clark, S. P. *et al. Nature* **311**, 387-389 (1984).
22. Davis, M. M. *et al. Nature* **283**, 733-739 (1980).
23. Leder, P. *et al. Science* **222**, 765-771 (1983).
24. Klein, G. *Nature* **294**, 313-318 (1981).
25. Seidman, J. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 6022-6026 (1980).
26. Coleclough, C., Perry, R. P., Karjalainen, K. & Weigert, M. *Nature* **290**, 372-378 (1981).
27. Nottenburg, C. & Weissman, I. L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 484-488 (1981).
28. Bernard, O., Gough, N. M. & Adams, J. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5812-5816 (1981).
29. Kwan, S., Max, E., Seidman, J. G., Leder, P. & Scharff, M. D. *Cell* **26**, 57-66 (1981).
30. Alt, F. W., Rosenberg, N., Enea, V., Siden, E. & Baltimore, D. *Molec. cell Biol.* **2**, 386-400 (1982).
31. Early, P. & Hood, L. *Cell* **24**, 1-3 (1981).
32. Altenburger, W., Steinmetz, M. & Zachau, H. G. *Nature* **287**, 603-607 (1980).
33. Max, E. E., Seidman, J. G., Miller, H. & Leder, P. *Cell* **21**, 793-799 (1980).
34. Early, P., Nottenburg, C., Weissman, I. & Hood, L. *Molec. cell Biol.* **2**, 829-836 (1982).
35. Sakano, H., Kurosawa, Y., Weigert, M. & Tonegawa, S. *Nature* **290**, 562-565 (1981).
36. Kurosawa, Y. *et al. Nature* **290**, 565-570 (1981).
37. Janeway, C. A. Jr., Wigzell, H. & Binz, H. *Scand. J. Immun.* **5**, 993-1001 (1976).
38. Reth, M. G. & Alt, F. W. *Nature* **312**, 418-423 (1984).
39. Goverman, J., Minard, K., Shastri, N., Hunkapiller, T., Hansburg, D., Sercarz, E. & Hood, L. *Cell* (in the press).
40. Alt, F. W. *et al. EMBO J.* **3**, 1209-1219 (1984).
41. Max, E. E., Seidman, J. G. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3450-3454 (1979).
42. Sakano, H., Huppi, K., Heinrich, G. & Tonegawa, S. *Nature* **280**, 288-294 (1979).
43. Steinmetz, M., Altenburger, W. & Zachau, H. G. *Nucleic Acids Res.* **8**, 1709-1720 (1980).
44. Selsing, E. & Storb, U. *Nucleic Acids Res.* **9**, 5725-5735 (1981).
45. Höchtli, J., Müller, C. R. & Zachau, H. G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1383-1387 (1982).
46. Van Ness, B. G., Coleclough, C., Perry, R. P. & Weigert, M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 262-266 (1982).
47. Lewis, S., Rosenberg, N., Alt, F. & Baltimore, D. *Cell* **30**, 807-816 (1982).
48. Höchtli, J. & Zachau, H. G. *Nature* **302**, 260-263 (1983).
49. Knight, K. L. & Hanly, W. C. *Contemp. Topics molec. Immun.* **4**, 55-88 (1975).
50. Hozumi, N. & Tonegawa, S. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3628-3232 (1976).
51. Lewis, S., Gifford, A. & Baltimore, D. *Nature* **308**, 425-428 (1984).
52. Kapp, J. A., Araneo, B. A. & Clevinger, B. L. *J. exp. Med.* **152**, 235-240 (1980).
53. Kapp, J. A. & Araneo, B. A. in *Isolation, Characterization and Utilization of T-Lymphocyte Clones* (eds Fathman, C. G. & Fitch, F. W.) 137-148 (Academic, New York, 1982).
54. Schibler, U., Marcu, K. B. & Perry, R. P. *Cell* **15**, 1495-1509 (1978).
55. Hieter, P. A., Korsmeyer, S. J., Waldmann, T. A. & Leder, P. *Nature* **290**, 368-372 (1981).
56. Toyonaga, B., Yanagi, Y., Suciu-Foca, N., Minden, M. & Mak, T. W. *Nature* **311**, 385-387 (1984).
57. Royer, H. D., Bensussan, A., Acuto, O. & Reinherz, E. L. *J. exp. Med.* **160**, 947-952 (1984).
58. Bensussan, A., Acuto, O., Hussey, R. E., Milanese, C. & Reinherz, E. L. *Nature* **311**, 565-567 (1984).
59. Germain, R. N. & Benacerraf, B. *Springer Semin. Immunopath.* **3**, 93-127 (1980).
60. Yoshikai, Y., Yanagi, Y., Suciu-Foca, N. & Mak, T. W. *Nature* **310**, 506-508 (1984).
61. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294-5299 (1979).
62. Kronenberg, M., Davis, M. M., Early, P. W., Hood, L. E. & Watson, J. D. *J. exp. Med.* **152**, 1745-1761 (1980).
63. Steinmetz, M. *et al. Nature* **300**, 35-42 (1982).

Structure of the calcium regulatory muscle protein troponin-C at 2.8 Å resolution

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Crystals of turkey skeletal muscle troponin-C reveal a molecule of two domains with an unusual structure. Two Ca^{2+} ions are bound to the C-terminal domain. The two cation-binding sites of the regulatory (N-terminal) domain are Ca^{2+} free; this domain adopts a markedly different conformation from the C-terminal domain. The two domains are connected by a long nine-turn α -helix; three of these turns are exposed fully to solvent.

THE crossbridge model of muscle contraction¹ proposes that the heads of myosin in the thick filaments cyclically attach and detach from the actin-containing thin filaments as ATP is hydrolysed. Hence the two sets of filaments move past one another in opposite directions and contractile force is generated. The contraction event is initiated by a nerve impulse that causes a

rapid increase in intracellular Ca^{2+} -ion concentration from $\sim 10^{-7}$ M in resting muscle to $\sim 10^{-5}$ M. In vertebrate skeletal and cardiac muscle, regulation of the contraction/relaxation cycle is at the level of the thin filaments, via the protein complex troponin/tropomyosin². The binding of Ca^{2+} ions to troponin-C (Tn-C) triggers a conformational change in the disposition of

the troponin/tropomyosin which may either remove a steric hindrance to the approach of the myosin head to actin³⁻⁵, or may allow the release of inorganic phosphate (P_i) from the actin/myosin head/ADP. P_i complex that is blocked in the relaxed state⁶.

The troponin complex consists of a Ca^{2+} -binding subunit (Tn-C), a subunit binding tightly to the 400-Å long coiled-coil tropomyosin molecule, troponin-T (Tn-T), and a subunit inhibiting the Mg^{2+} -activated ATPase of actomyosin, troponin-I (Tn-I)⁷, all of known amino-acid sequence⁸⁻¹⁰. Here, we describe turkey skeletal muscle Tn-C and attempt to relate its recently determined molecular structure to its role in muscle contraction.

Tn-C has four Ca^{2+} -binding sites¹¹, two with high affinity (association constant (K_a) $\sim 10^7 M^{-1}$) that bind Mg^{2+} competitively, and two with lower affinity ($K_a \sim 10^5 M^{-1}$) specific for Ca^{2+} and in the N-terminal domain of the molecule. These latter sites control the regulatory function of the protein. This is in contrast to an evolutionary related Ca^{2+} -binding protein, calmodulin, in which the binding affinity of the four sites are closer to being equivalent^{12,13}. Crystals of this important regulatory protein have been reported¹⁴⁻¹⁶. From a comparison of amino-acid sequences of the several Tn-C molecules so far studied (the amino-acid sequence for turkey Tn-C has not been reported but its amino acid composition is similar to that of chicken Tn-C¹⁷) with the sequence of carp parvalbumin, the four Ca^{2+} -binding regions of the Tn-C molecule have been identified⁸. Each of these regions most probably adopts the supersecondary structure of helix-loop-helix so familiar in the family of Ca^{2+} -binding proteins¹⁸. This motif has been termed the EF-hand after one of the Ca^{2+} -binding domains of parvalbumin. Indeed, based on the structure of carp parvalbumin, several predictions of the tertiary structure of Tn-C have been made^{19,20}. Here, we evaluate these predictions.

Molecular conformation of Tn-C

Details of the structure determination are given in Fig. 1 legend. Figure 2a shows the folding of Tn-C and Fig. 2b a stereo representation of the α -carbon chain tracing. The molecule is dumbbell-shaped, 75 Å long with $\sim 67\%$ of the amino acids in helical conformation. The exact residue number for the start and end of a helical segment may change following the full least-squares refinement of the structure at 2.2 Å resolution.

Tn-C consists of two domains joined by a nine-turn helix with no direct intramolecular interactions between the domains. The distance between the centres of mass of the two domains is ~ 40 Å. Although our crystals are obtained from solutions containing excess Ca^{2+} ions sufficient to fill all four Ca^{2+} -binding sites, only the two high-affinity sites of the C-terminal domain (III and IV) are occupied by Ca^{2+} in our crystal form.

The N-terminal domain (N-domain) includes the first 85 amino-acid residues. The positions of the first two residues are not determined as the associated electron density is poorly defined. The second domain (C-domain) comprises residues Glu 97–Gln 162, of which the position of the last residue is undefined. The N-terminal polypeptide chain starts in a helical stretch for residues Ala 4–Ala 12, then bends and continues through four additional helices that adopt the supersecondary disposition helix-loop-helix-linker-helix-loop-helix. The four helices A–D span the following residues: A, Glu 16–Phe 29; B, Lys 40–Met 48; C, Lys 55–Asp 66; D, Phe 75–Gln 85. Loops I and II (Fig. 2a) correspond to the empty low-affinity Ca^{2+} -binding sites. The connection between the two domains is made via a helical linker (D/E) from helix D of the N-domain to helix E of the C-domain, thus forming the long continuous nine-turn helix spanning residues Phe 75–Asp 106. This is a slightly bent helix, such that the angle between the D and the E helix axes is $\sim 10^\circ$. The C-domain contains four helices, E, F, G and H: E, Glu 96–Asp 106; F, Ile 115–Thr 125; G, Glu 131–Asp 142; H, Phe 151–Gln 159. Loops III and IV (Fig. 2a) are occupied by Ca^{2+} .

Considering the helix-loop-helix as the building unit from which Tn-C is constructed, there is a non-crystallographic

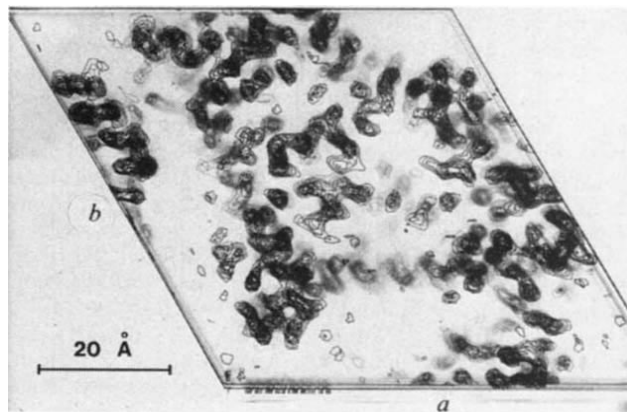


Fig. 1 Eleven sections of the 2.8-Å resolution, figure of merit weighted electron-density map of Tn-C (sections from $z = -0.045$ to $z = 0.106$). Crystals of turkey skeletal muscle Tn-C are obtained from solutions of 39–42% saturated $(NH_4)_2SO_4$, 3–5% (v/v) polyethylene glycol 200, 10 mM $CaCl_2$ and 100 mM sodium acetate buffer, pH 5.0 (ref. 36). The crystals are trigonal with space group $P3_221$ and unit cell dimensions of $a = b = 66.55(2)$, $c = 60.90(2)$ Å and $\gamma = 120^\circ$. X-ray intensity data to 2.8 Å resolution for the native protein and 11 isomorphous heavy-atom derivatives were collected with an Enraf-Nonius CAD4 diffractometer using a data collection protocol described previously³⁷. Heavy-atom binding sites were determined from difference Patterson maps and/or difference Fourier maps in the usual manner³⁸. The positional parameters, isotropic temperature factors and occupancies for the heavy-atom derivatives were refined by lack of closure error minimization³⁹ using Victor, a computer program originally written by M. G. Rossmann and modified by us. The results of the multiple isomorphous replacement (MIR) phase determination for Tn-C are given in Table 1. A 2.8-Å-resolution electron-density map was computed using the centroid phases⁴⁰ and measured structure factor amplitudes modified by the figures of merit. The overall figure of merit for the observed 3,877 reflections (95% of the data to 2.8 Å resolution) was 0.90 and the resultant quality of the map is very high. The sections shown above contain the density for a nine-turn α -helical segment of Tn-C running parallel to the b crystallographic axis. Part of the same nine-turn α -helix of a neighbouring molecule, symmetry related to the first by a 2-fold rotation axis parallel to the cell diagonal at $z = 0$, can be seen running parallel to the a axis. Contours on these sections start at $0.3 e \text{ Å}^{-3}$ and are in steps of $0.2 e \text{ Å}^{-3}$.

pseudo-2-fold rotation axis in each domain that relates pairs of building units: AB to CD and EF to GH. The difference in the spatial appearance of the two domains is due not only to the presence of the extra N-terminal helix in the N-domain, but also to the differences in the inter-helix angles between pairs of helices of each Ca^{2+} -binding unit. The helices in the N-domain tend to be antiparallel, forming a non-ideal up-and-down antiparallel helix bundle²¹. This is not the case in the C-domain, where the helices are arranged in a manner closely similar to the CDEF region of parvalbumin¹⁸.

Ca^{2+} -binding loops

There is no electron density for Ca^{2+} ions in the region of the two loops, I and II, of the Ca^{2+} -specific domain (Fig. 3a, b), thus it is not yet known exactly which residues in these loops are the coordinating ligands. Based on sequence homologies with other Ca^{2+} -binding proteins²², the following residues may be ligands: Asp 30, Asp 32, Gly 34 (possibly via the main-chain carbonyl oxygen or a water molecule), Asp 36, Ser 38 and Glu 41 in loop I and Asp 66, Asp 68, Ser 70, Thr 72 (main-chain carbonyl oxygen), Asp 74 and Glu 77 in loop II. The proposed ligands in loop I would result in a departure from the model Ca^{2+} -binding loop conformation of parvalbumin¹⁸. In this archetype, only the fourth ligand is a main-chain carbonyl oxygen atom. In Tn-C, a glycine is in the third position and

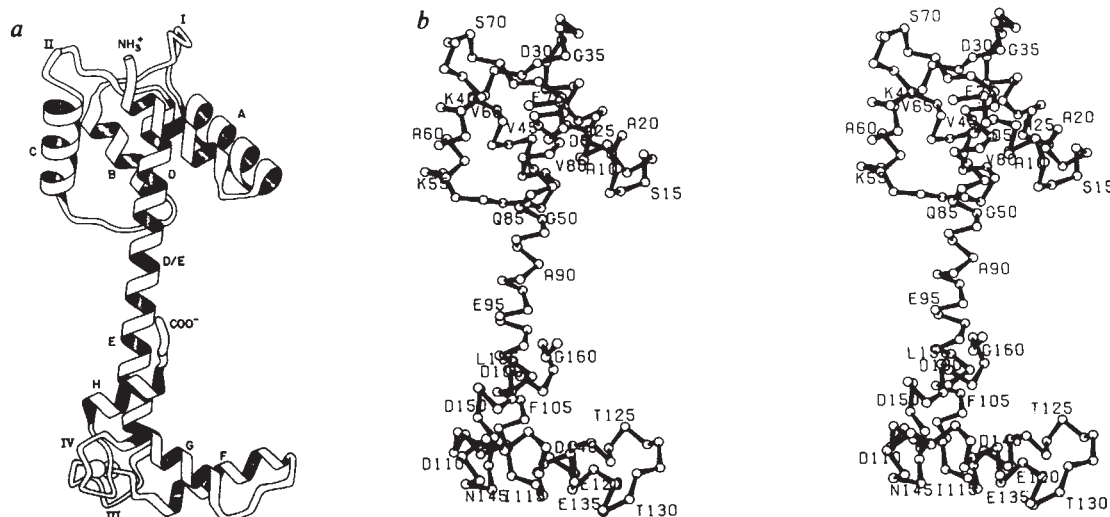


Fig. 2 Tn-C crystal structure fold. The complete polypeptide chain has been traced unambiguously and the amino acid side chains have been fitted according to the known sequence of chicken Tn-C¹⁴. All the model interpretation was done directly on an MMS-X interactive computer graphics system with the M3 program of Colin Broughton⁴¹. The resulting molecular geometry has been refined by the least-squares procedure of Hendrickson and Konner⁴². The present R -factor ($=\sum ||F_o|-|F_c||/\sum |F_o|$) is 0.22 for the 2.8-Å resolution data. The α -carbon coordinates have been deposited in the Brookhaven Protein Data Bank⁴³. **a**, Ribbon representation of the polypeptide chain of turkey skeletal muscle Tn-C. The upper helical domain is the calcium regulatory domain; the lower domain is the high-affinity $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding domain. Helices corresponding to homologous helical regions in parvalbumin¹⁸ are labelled A-H sequentially. There is an additional helical segment in the N-terminal region of Tn-C. The segments of polypeptide chain forming the Ca^{2+} -binding loops are labelled I to IV sequentially. Ca^{2+} ions are found only in sites III and IV. **b**, Stereo representation of Tn-C showing the α -carbon positions in the molecule. The molecule is shown in a similar orientation to that in **a** and every fifth amino acid position is labelled. The Ca^{2+} ions have been omitted for clarity.

thus there is no side chain to bind calcium. The coordination provided by the fourth position, Asp 36, could be either from the side-chain carboxylate or from the main-chain carbonyl oxygen.

The coordinating ligands for the Ca^{2+} ions in loops III and IV (Fig. 3c, d) are as follows: loop III, Asp 106, Asn 108, Asn 110, Phe 112 (main-chain carbonyl oxygen), Asp 114 and Glu 117; loop IV, Asp 142, Asn 144, Asp 146, Arg 148 (main-chain carbonyl oxygen), Asp 150 (not directly) and Glu 153. Figure 3d shows that the side chain of Asp 150 is 6.3 Å from the Ca^{2+} ion. In our interpretation of the structure, this side chain could only be coordinated to the Ca^{2+} ion via an intervening water molecule. The Ca^{2+} ions are separated by 11.3 Å.

Comparison of the conformations of loops I and II with those of loops III and IV in which Ca^{2+} ions are bound shows the kinds of conformational changes in loops I and II that must accompany Ca^{2+} binding. In particular, these loops must adjust in order to bring Glu 41 and Glu 77 as close to their respective Ca^{2+} ions as seen for the side chains of Glu 117 and Glu 153. These changes could bring about further movements of helices to make the N-terminal domain closer in conformation to the $\text{Ca}^{2+}/\text{Mg}^{2+}$ domain.

$\text{Ca}^{2+}/\text{Mg}^{2+}$ domain

The inter-helix angle between the N-helix and C-helix of Ca^{2+} -binding sites, together with the conformation of the Ca^{2+} -ion ligands, provide a measure of the extent to which each of these

Table 1 Statistics for multiple isomorphous replacement phasing of troponin-C

Heavy atom	Soaking time (h)	Resolution used (Å)	No. unique reflections used	R_{iso} (2.8 Å)¶	No. of sites	R_{cen} ¶	$\langle E_H \rangle / \langle f_H \rangle$ ¶
1 mM Mersalyl acid	18	2.8	3,846	0.22	2 [§]	0.36	0.33
1 mM $\text{Hg}(\text{SCH}_2\text{CH}_2\text{NH}_3)_2\text{Cl}_2$	19	2.8	2,877	0.19	2 [§]	0.47	0.47
0.1 Sat. PCMBs*	24	2.8	3,877	0.25	1 [§]	0.43	0.39
0.25 Sat. DCMNP†	36	2.8	3,877	0.11	2 [§]	0.62	0.64
0.1 Sat Mercury chloranilate	20	2.8	3,877	0.23	1 [§]	0.37	0.31
5 mM OsCl_3	24	4.5	953	0.26	1 [§]	0.36	0.33
1 mM $\text{K}_3\text{UO}_2\text{F}_5$ ‡	19	2.8	3,849	0.24	3	0.46	0.42
1 mM Mersalyl acid + 1 mM $\text{K}_3\text{UO}_2\text{F}_5$	24	2.8	3,869	0.30	5 [§]	0.41	0.39
5 mM TmCl_3	16	2.8	3,877	0.21	2	0.47	0.50
1 mM LuCl_3	20	2.8	3,876	0.18	3	0.54	0.50
1 mM EuCl_3	18	4.5	971	0.20	3	0.33	0.31

* PCMBs, *p*-chloromercuribenzenesulphonate; † DCMNP, 2,6-dichloromercuri-4-nitrophenol; ‡ anomalous dispersion data used in phasing.

§ The mercury derivatives and OsCl_3 have one or two common sites between Cys 101 of one molecule and Met 28 of a neighbouring one. Such pairs of common sites are 4 Å apart, coordinating to the methionine side chain from opposite directions.

|| These three heavy-atom derivatives are lanthanides, known to have higher affinity to Ca^{2+} -binding sites than Ca^{2+} itself. Of these, Tm^{3+} was the most isomorphous derivative and Eu^{3+} the least. Eu^{3+} and Lu^{3+} replaced Ca^{2+} in binding sites III and IV and Tm^{3+} replaced Ca^{2+} in site III only. Another site was found outside of loop I. The lanthanides in site III are bound within 1 Å of the Ca^{2+} position, whereas in IV they are found 5.5 Å away from the Ca^{2+} position. Further refinement of the structure will enable us to analyse the mode of lanthanide binding in more detail.

¶ $R_{\text{iso}} = \frac{\sum_h ||F_{\text{PH}}^{\text{obs}}| - |F_{\text{P}}^{\text{obs}}||}{\sum_h |F_{\text{P}}^{\text{obs}}|}$ $R_{\text{cen}} = \frac{\sum_{h \text{ centric}} ||F_{\text{PH}}^{\text{obs}}| - |F_{\text{P}}^{\text{obs}}| + F_{\text{H}}^{\text{calc}}|}{\sum_{h \text{ centric}} ||F_{\text{PH}}^{\text{obs}}| - |F_{\text{P}}^{\text{obs}}||}$
 $\langle E_H \rangle$, r.m.s. lack of closure error; $\langle f_H \rangle$, r.m.s. heavy-atom scattering.

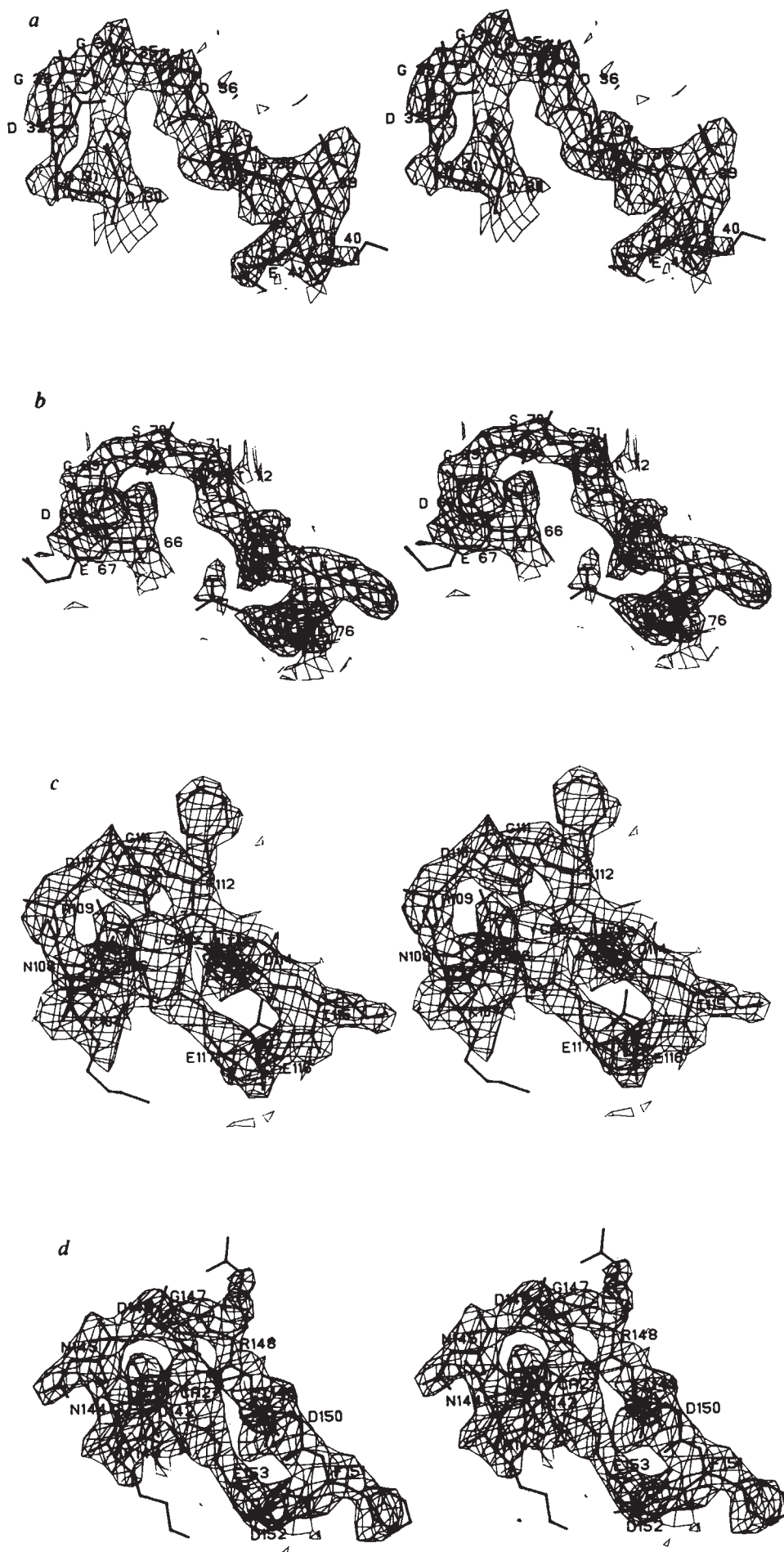
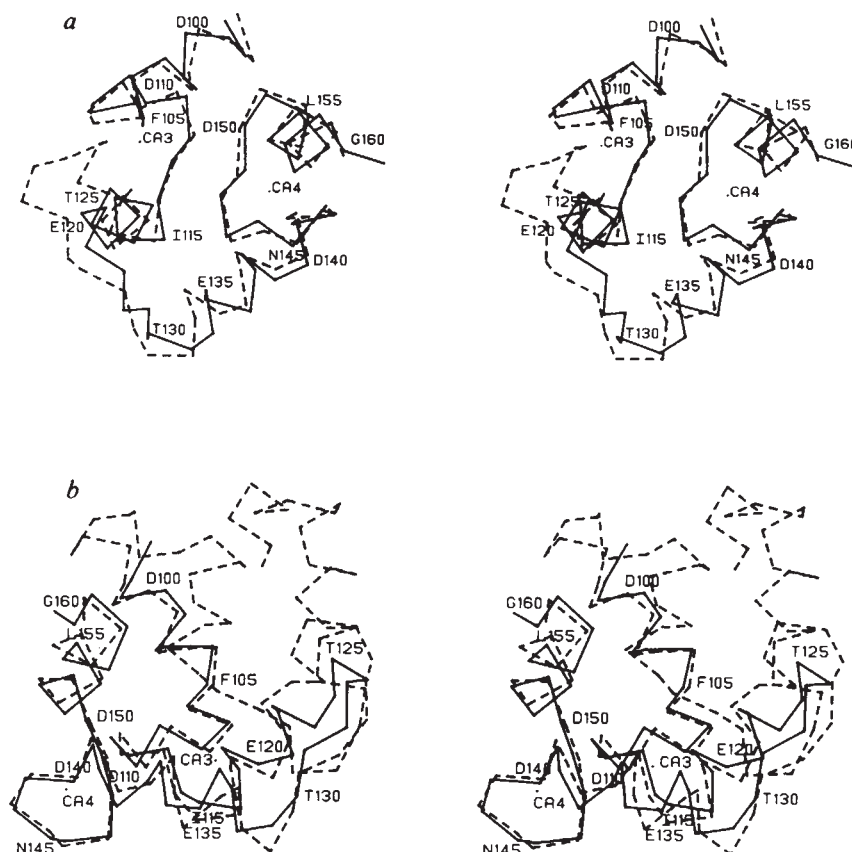


Fig. 3 Stereoscopic representations of the four Ca^{2+} -binding loops in Tn-C: *a-d* correspond to Ca^{2+} -binding sites I to IV, respectively. All loops have been oriented so they present similar aspects to the viewer; in particular the glutamate residue in the sixth coordinating ligand position is in the bottom right of each figure. Superimposed on the molecular model is the electron density for each loop from the 2.8-Å resolution MIR map. The contour level for *a* and *b* is $0.30 \text{ e } \text{\AA}^{-3}$, that for *c* and *d* is $0.40 \text{ e } \text{\AA}^{-3}$. Some of the side chains have relatively weak electron density in the MIR map: Asp 32, Lys 40, Glu 67, Lys 107, Glu 116, Lys 143, Arg 148 and Asp 152.

Fig. 4 The CDEF domain of carp parvalbumin¹⁸ (dashed line) superimposed on the EFGH region of Tn-C (solid line). *a*, This stereoscopic representation of α -carbon positions is drawn looking approximately along the non-crystallographic pseudo-2-fold rotation axis relating the two Ca^{2+} -binding sites III and IV. Residues of the Tn-C molecule are labelled with the residue name and sequence number based on the chicken Tn-C sequence¹⁷. The major structural departure of the two linker regions is due in part to a five-residue insertion in parvalbumin over Tn-C. *b*, The same superposition of the C-terminal domain of Tn-C with the CDEF domain of parvalbumin. Residues of the AB segment of parvalbumin are also included. This view is approximately orthogonal to that in *a* and shows that the AB segment would fit into a markedly concave surface in Tn-C between the EH and FG helices.



helix-loop-helix motifs in Tn-C resemble the archetypal EF hand¹⁸. In parvalbumin, the inter-helix angles for the CD and EF binding domains are 103 and 102°, respectively. Similarly, both inter-helix angles of the EF and GH helices in the C-domain of Tn-C are 107°. This similarity of the two proteins is substantiated further by the fact 56 of the 66 α -C atoms of the C-domain of Tn-C can be superposed on the CDEF domain of parvalbumin with a root-mean-square deviation between the paired α -carbon coordinates of 1.25 Å (Fig. 4*a*). The residues that fit best span helices E and H, the two Ca^{2+} -binding loops and parts of helices F and G in Tn-C. The departure from the conformation of parvalbumin accumulates gradually in the F and G helices and is very prominent in the linker region between the two binding sites. This is a consequence of two features: the parvalbumin linker is five residues longer than the corresponding segment in turkey skeletal Tn-C and helix D in parvalbumin is bent, in contrast to the corresponding F helix in Tn-C.

The resemblance of the high-affinity domain of Tn-C to the bovine intestinal Ca^{2+} -binding protein²³ is less striking. In the latter protein, the inter-helix angles are different, 121° for the helices of both binding sites, and the geometry of the first Ca^{2+} -binding loop deviates from the archetypal loop markedly, due to an insertion of an additional residue between the third and fourth ligand and the fact that the first three coordinating ligands are main-chain carbonyl oxygen atoms. The fold of the linker is also different in these two proteins. In Tn-C it has an extended conformation (Fig. 4*a*), but the intestinal protein has a linker with a single helical turn.

Ca^{2+} -specific regulatory domain

The N-domain of Tn-C in the present crystal form is Ca^{2+} free (Fig. 3*a, b*). Presumably due to the lack of Ca^{2+} , the paired helices AB and CD have markedly different inter-helix angles from those of the C-domain. The angle between helices A and B is 145° whereas that between helices C and D is 150°. An additional feature of this domain lacking in the C domain is the helix at the N-terminus (Fig. 2). Relative shifts of helices,

which could occur on Ca^{2+} binding, might also involve the reorientation of this helix.

The crystal structure shows that helix D is surrounded completely by the other four helices of the N-domain. It is unusual for a helix to be buried completely in a hydrophobic core, but the hydrophobic character of helix D (the sequence from residue 75 is Phe-Glu-Glu-Phe-Leu-Val-Met-Met-Val-Arg-Gln) is consistent with its environment. Ten of these 11 residues are buried mostly or completely with only Arg 84 exposed to solvent. There are two salt bridges providing additional stability to the way these helices pack, one between Arg 84 of helix D and Glu 64 of helix C and the other between Glu 76 of helix D and Arg 11 of the N-terminal helix. The stabilization associated with the arrangement of the five helices when Ca^{2+} is not bound to the regulatory domain may be in part responsible for the reduced affinity for Ca^{2+} ions of sites I and II.

N- and C-domain hydrophobic cores

The structure of Tn-C described here provides data on the conformations of Ca^{2+} -bound helix-loop-helix motifs and Ca^{2+} -free motifs. It has been suggested that certain hydrophobic residues which may be buried in the Ca^{2+} -free conformation become exposed when Ca^{2+} binds, thus enhancing the binding of some neuroleptic drugs (such as trifluoperazine) to Ca^{2+} -saturated Tn-C. Binding studies to the C-terminal half of rabbit skeletal Tn-C²⁴ indicate that the drug binds to helix E via an interaction with Phe 99 and Phe 102 (homologous to residues 102 and 105 in turkey skeletal Tn-C). In the present crystal structure of turkey Tn-C, each domain has a cluster of four phenylalanine residues in its respective hydrophobic core. In the N-domain these residues are on helices A and D: Phe 22, Phe 26, Phe 75 and Phe 78; those in the C-domain are on helices E and H, Phe 102, Phe 105, Phe 151 and Phe 154. Seven of these side chains are buried, only Phe 151 is exposed. Thus, in the Ca^{2+} -bound state, the buried side chains of Phe 102 and Phe 105 on the E helix cannot interact directly with trifluoperazine unless such drugs perturb the structure significantly.

Exposed helical linker D/E

One of the most unusual features of the Tn-C molecule is the long nine-turn α -helix running from the N-domain to the C-domain. A segment of this helix, consisting of 11 amino acids, is largely solvated such that only seven atoms have intermolecular contacts <4.0 Å and there are no intramolecular tertiary contacts less than this value. This segment has the following sequence: Lys 87-Glu-Asp-Ala-Lys-Gly-Lys-Ser-Glu-Glu-Glu, the central glycine flanked on either side by a lysine residue, a neutral residue and then three charged residues. Most of the residues are of relatively high helix-forming propensity with the exceptions of Gly 92 and Ser 94. Gly residues are associated with regions of flexibility in a polypeptide chain²⁵, which suggests that the helix linker might adopt different conformations in the region of Gly 92 that could give rise to a fold in which the Ca^{2+} -specific regulatory domain interacts directly with the $\text{Ca}^{2+}/\text{Mg}^{2+}$ domain. We have explored such conformational changes using interactive molecular graphics; they are easy to achieve with only two rotatable bonds, ϕ and ψ , of Gly 92. Other conformations for this helical region are also possible, including partial or full unwinding of the linker region due to changes in the electrostatic environment associated with Ca^{2+} binding²⁶.

The possibility that this extended conformation for Tn-C is the result of intermolecular packing forces has to be considered. The crystal packing of Tn-C places the N-domain of a symmetry-equivalent molecule (related by the 3_2 screw axis) up against the concave surface between helix pairs EH and FG of the C-domain (Fig. 2a, b). In parvalbumin, the equivalent space is occupied by the AB helical region of the degenerate Ca^{2+} -binding site (Fig. 4b). Figure 5 shows how these two symmetry-related molecules in Tn-C interact. As the exposed segment of the helical linker, Lys 87 to Glu 97, makes so few direct intermolecular contacts, its extended helical conformation may be stable independently and not determined by the crystal packing.

Physiological function of Tn-C

The crystal structure determination of turkey skeletal muscle Tn-C presented here raises questions about the relevance of its tertiary structure to that in muscle, where it functions as part of the troponin complex. The lack of Ca^{2+} binding in the Ca^{2+} -regulatory domain could be related to the relatively low pH (5) of the crystals. Either the proton concentration is sufficient to compete with Ca^{2+} for the low-affinity sites or there is additional stabilization provided to the N-domain, trapping its conformation in the apo-state. Solution measurements of the pH dependence of the CD and fluorescence spectra of Ca^{2+} -free Tn-C show changes similar to those of Ca^{2+} titrations^{27,28}. These have been associated with the neutralization of carboxyl groups with abnormally high pK_{as} ~6. Similar observations have been made by monitoring the pH dependence of Ca^{2+} binding to the regulatory domain with ^{43}Ca -NMR²⁹. Four features of this crystal structure are pH dependent: (1) close intramolecular interactions between Glu 57 in helix C and Glu 88 in helix D; (2) close interactions between Glu 131 and Glu 135 in helix G; (3, 4) the intermolecular interactions between Glu 132 and Asp 136 of helix G with Asp 136 and Glu 132 respectively of a neighbouring molecule, symmetry related by 2-fold rotation. Such interactions have been found in many protein structures³⁰ and are attributed to the sharing of a proton by the two negatively charged side chains. Pairs of this type are associated with one low and one abnormally high pK_{a} ~6. The intermolecular carboxyl/carboxylate pairs provide an explanation for the extreme sensitivity of the crystals to pH. Whatever the role of pH, clearly the conformation of the Ca^{2+} -free loops is very different from Ca^{2+} -occupied loops. The N-domain loop conformations may be more like the state in relaxed muscle. The C-domain is always saturated with Ca^{2+} or Mg^{2+} in physiological conditions. Therefore, we believe that its structure in the troponin complex will not differ significantly from that determined crystallographically.

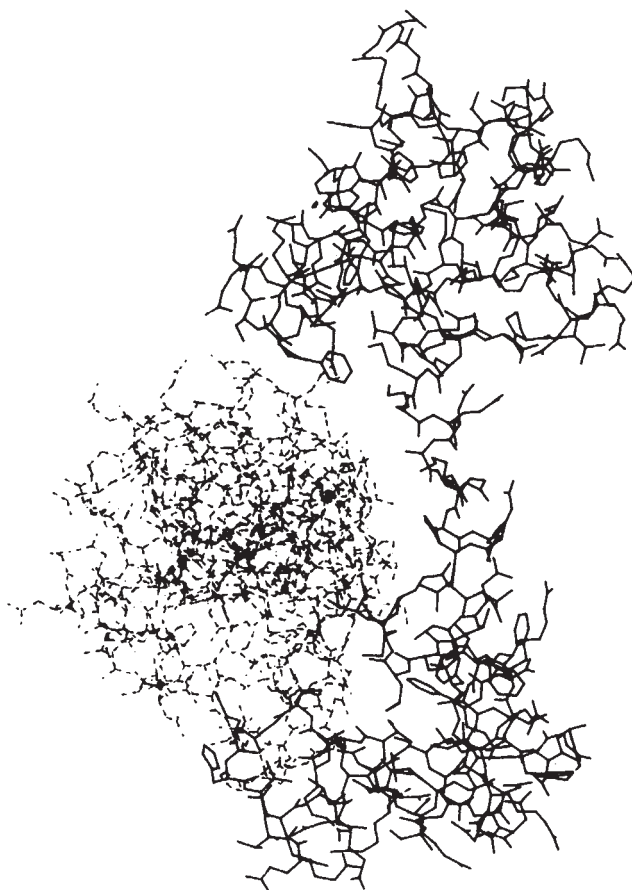


Fig. 5 A view of the whole Tn-C molecule from an aspect 180° from that shown in Fig. 2. The molecule in dashed lines is related to the one in solid lines by a 3_2 crystallographic axis and thus shows the intermolecular packing interaction between the N-domain of the dashed line molecule and the C-domain of the molecule drawn in solid lines. Not many of the residues of the D/E helical linker interact directly with residues of the symmetry-related molecule. More interactions are found in the region of helices F, G and H.

The differences in the Ca^{2+} affinities of the two domains may be attributed both to the factors that stabilize the buried helix D as discussed before and to the differences between the amino-acid sequences and the nature of Ca^{2+} ligands in the loops of each domain.

It is not yet clear whether the unusual structure of Tn-C in this crystal form, in which there is a complete separation between the two domains via the helical linker, is relevant to the structure of Tn-C in the troponin complex. There are conflicting results from solution studies of Tn-C concerning the presence or absence of interaction between the domains³¹⁻³⁴, no such evidence exists for the complex. The hypothetical conformational change in the helix-linker region on Ca^{2+} binding would bring about a change in the relative disposition of the two domains, both of which interact with Tn-I³⁵. This implies a change in the relative position of Tn-I that would remove its inhibitory effect and allow the process of contraction to begin. Alternatively, changing the inter-helix angles in the regulatory domain might destabilize the interaction of this domain with Tn-I, or could contribute to a change in the electrostatic conditions destabilizing the D/E helix linker.

Finally, we can now evaluate the success of previous structure predictions of Tn-C. The helix-loop-helix supersecondary structural fold for the EF and GH regions of Tn-C was predicted correctly by Kretsinger and Barry¹⁹. The tertiary structural

arrangement of the Ca^{2+} -regulatory domain, however, differs from their prediction. This may be due to the lack of Ca^{2+} in our structure; indeed when Ca^{2+} is bound in sites I and II, this domain could rearrange to more closely resemble the C-domain. The overall shape of this predicted structure is very different from that in the crystal because of the failure to predict the unique nine-turn helical linker and the additional N-terminal

helix. The assumption of Nagano and Ohtsuki²⁰ that the inter-helix angles should be 0° turns out to be incorrect.

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- Huxley, H. E. *Science* **164**, 1356–1366 (1969).
- Adelstein, R. S. & Eisenberg, E. A. *Rev. Biochem.* **49**, 921–956 (1980).
- Haselgrove, J. C. *Cold Spring Harb. Symp. quant. Biol.* **37**, 341–352 (1973).
- Huxley, H. E. *Cold Spring Harb. Symp. quant. Biol.* **37**, 361–376 (1973).
- Wakabayashi, T., Huxley, H. E., Amos, L. A. & Klug, A. *J. molec. Biol.* **93**, 477–497 (1975).
- Chalovich, J. M., Chock, P. B. & Eisenberg, E. *J. biol. Chem.* **256**, 575–578 (1981).
- Greaser, M. L. & Gergely, J. *J. biol. Chem.* **248**, 2125–2133 (1973).
- Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G. & Jackman, N. *FEBS Lett.* **36**, 268–272 (1973).
- Pearlstone, J. R., Johnson, P., Carpenter, M. R. & Smillie, L. B. *J. biol. Chem.* **252**, 983–989 (1977).
- Wilkinson, J. M. & Grand, R. J. A. *Biochem. J.* **149**, 493–496 (1975).
- Potter, J. D. & Gergely, J. *J. biol. Chem.* **250**, 4628–4633 (1975).
- Gouch, T. H. & Klee, C. B. *Biochemistry* **19**, 3692–3698 (1980).
- Deville, A., Grandjean, J., Laszlo, P., Brzeska, H. & Drabikowski, W. *Eur. J. Biochem.* **109**, 515–522 (1980).
- Cook, W. J., Dedman, J. R., Means, A. R. & Bugg, C. E. *J. biol. Chem.* **255**, 8152–8153 (1980).
- Kretsinger, R. H., Rudnick, S. E., Sneden, D. A. & Schatz, V. B. *J. biol. Chem.* **255**, 8154–8156 (1980).
- Gehrig, L. M. B., Delbaere, L. T. J. & Hickie, R. A. *J. molec. Biol.* **177**, 559–561 (1984).
- Wilkinson, J. M. *FEBS Lett.* **70**, 254–256 (1976).
- Kretsinger, R. H. & Nockolds, C. E. *J. biol. Chem.* **248**, 3313–3326 (1973).
- Kretsinger, R. H. & Barry, C. D. *Biochim. biophys. Acta* **405**, 40–52 (1975).
- Nagano, K. & Ohtsuki, I. *Proc. Japan. Acad. B* **58**, 73–77 (1982).
- Richardson, J. *Adv. Protein Chem.* **34**, 167–339 (1981).

- Tufty, R. M. & Kretsinger, R. H. *Science* **187**, 167–169 (1975).
- Szebenyi, D. M. E., Obendorf, S. K. & Moffat, K. *Nature* **294**, 327–332 (1981).
- Gariépy, J. & Hodges, R. S. *Biochemistry* **22**, 1586–1594 (1983).
- Huber, R. *Trends biochem. Sci.* **4**, 271–276 (1979).
- Hol, W. G. J., van Duijnen, P. Th. & Berendsen, H. J. C. *Nature* **273**, 443–446 (1978).
- Lehrer, S. S. & Leavis, P. C. *Biochem. biophys. Res. Commun.* **58**, 159–165 (1974).
- Hinck, M. T., McCubbin, W. D. & Kay, C. M. *Can. J. Biochem.* **56**, 384–395 (1978).
- Anderson, T. thesis, Univ. Lund (1981).
- Sawyer, L. & James, M. N. G. *Nature* **295**, 79–80 (1982).
- Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z. & Drabikowski, W. *J. biol. Chem.* **253**, 5452–5459 (1978).
- Seamon, K. B., Hartshorne, D. J. & Bothner-By, A. A. *Biochemistry* **16**, 4039–4046 (1977).
- Levine, B. A., Mercola, D., Thornton, J. M. & Coffman, D. J. *J. molec. Biol.* **115**, 743–760 (1977).
- Brzeska, H., Vennyaminov, S. V., Grabarek, Z. & Drabikowski, W. *FEBS Lett.* **153**, 169–173 (1983).
- Grabarek, Z. & Drabikowski, W. *J. biol. Chem.* **256**, 13121–13127 (1981).
- Herzberg, O., Hayakawa, K. & James, M. N. G. *J. molec. Biol.* **172**, 345–346 (1984).
- James, M. N. G., Sielecki, A., Salituro, F., Rich, D. H. & Hofmann, T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6137–6141 (1982).
- Blundell, T. L. & Johnson, L. N. *Protein Crystallography* (Academic, London, 1976).
- Dickerson, R. E., Weinzierl, J. E. & Palmer, R. A. *Acta crystallogr.* **B24**, 997–1003 (1968).
- Blow, D. M. & Crick, F. H. C. *Acta crystallogr.* **12**, 794–802 (1959).
- Sielecki, A. R., James, M. N. G. & Broughton, C. G. in *Proc. Int. Summer Sch. Crystallogr. Computing* (ed. Sayre, D.) 409–419 (Oxford University Press, 1982).
- Hendrickson, W. A. & Konnert, J. H. in *Biomolecular Structure, Function, Conformation and Evolution* Vol. 1 (ed. Srinivasan, R.) 43–57 (Pergamon, Oxford, 1980).
- Bernstein, F. C. *et al. J. molec. Biol.* **112**, 535–542 (1977).

Optical pulsations in the Large Magellanic Cloud remnant 0540–69.3

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The X-ray pulsar PSR0540–693 was discovered in the Large Magellanic Cloud (LMC) supernova remnant, 0540–69.3, by Seward, Harnden and Helfand¹, as a pulse, with repetition period ~ 50 ms, in Einstein Observatory data. Previously, Clark *et al.*² had noted that this remnant resembles the Crab Nebula because of the X-ray power law spectrum and suggested that the nebular emission was synchrotron radiation powered by a central pulsar. After the announcement of X-ray pulsed emission, Chanan *et al.*³ measured the broad optical band properties of the nebula and found evidence for synchrotron emission. They reported that the 4.5-arcsec continuum emission remnant has only a tenth of the luminosity of the Crab Nebula. We have now detected pulsed optical emission for the X-ray pulsar, having a time-averaged magnitude of ~ 22.7 .

We recorded broad-band optical time-series data at 1-ms intervals with the 4-m and 1.5-m Cerro Tololo telescopes and have found strong pulsations, using the usual Fourier transform methods^{4,5}. Table 1 gives a summary of the observations, including magnitudes, barycentric frequencies and times of arrival. The pulse profile is predominantly sinusoidal with a 10–20% dip in the intensity maximum (see Fig. 1). Although this dip persists in the three most sensitive time series, the amplitudes of the next highest harmonics (60 and 80 Hz) correspond to

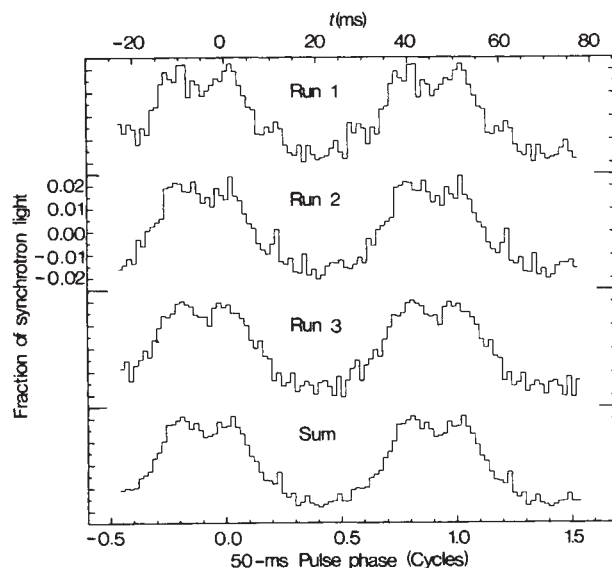


Fig. 1 The optical pulse profiles of the LMC PSR0540–693 from runs 1–3 (see Table 1) and the average pulse profile of these runs are shown repeated over two whole cycles. The profiles have been co-aligned according to the phase of the 20-Hz fundamental structure.

only 12 and 14% of the amplitude at the fundamental 20 Hz frequency. The average pulse profile for these runs (Fig. 1, lowest) shows that the duty cycle of the pulse is $\sim 44\%$, not 50%. No significant 10-Hz structure (at half the fundamental frequency) was found in the time series data, the corresponding amplitude having a 90% probability of being $< 10\%$ of the 20-Hz amplitude.

Table 1 Log of time series observations of PSR0540-693

Run number	1	2	3	4	5
UT August 1984	26.38182241(1)	27.36440074(1)	28.3595*	30.36588796(2)	31.34988462(1)
Duration (h)	1.39	2.28	2.33	1.78	2.12
Telescope	4-m	4-m	4-m	1.5-m	1.5-m
Wavelength (nm)	320-750†	320-750	320-750	350-900‡	350-900
Aperture diam. (arcs)	4.6	4.6	6.6	7.0	7.0
Pulse freq. _⊙ (Hz)	19.8881615(64)	19.8881420(26)	19.8881242(33)	19.888101(12)	19.8880695(73)
Pulse period _⊙ (ms)	50.281168(16)	50.281218(7)	50.281263(8)	50.281322(30)	50.281401(18)
(B + V)/2	22.75(0.06)§	22.65(0.04)	22.75(0.05)	22.33(0.15)	22.34(0.10)
Mean epoch (August 1984)		28.8659			
Mean frequency _⊙		19.8881152(16 Hz = 50.2812856(40) ms			
Mean $\partial f/\partial t$		-2.05(0.11) $\times 10^{-10}$ Hz s ⁻¹ = 44.8(2.4) ns day ⁻¹			

* No accurate time of arrival could be measured for this run.

† This corresponds to the bandpass of the atmosphere and an S20 photocathode extended by prismatic reflection.

‡ This is the bandpass of a GaAs photocathode.

§ These errors apply only to the inter-comparison of runs 1-3.

|| A colour correction of 0.39(B - V) must be applied to the (B + V)/2 values for runs 4 and 5.

The symbol $\odot$ here denotes a barycentric quantity.

The integrated broadband pulsed light intensity $\sim (B + V)/2$ ($B = \text{blue}$; $V = \text{visual}$ —magnitudes), is approximately equivalent to $(22.7 \text{ mag}) \times \sin(2\pi 20t)$, corresponding to just under 2% of the nebular light included in the 4.6-arc s circular aperture used in runs 1 and 2. This circular aperture was centred by count rate on the bright knot of the synchrotron emission, 1 or 2 arc s west of the centre of the OIII emission ring surrounding the nebula³. The centering was achieved by adjusting the offsets of a star-tracking autoguider probe that controlled the telescope motion during the data recording interval. The lack of erratic modulation of the pulse amplitude and the consistency of the magnitudes derived in runs 1-3 implies that the pulsing source coincides with the bright peak of the continuum emission³ to within 1.5 arc s.

The intrinsic optical luminosity of the LMC pulsar ($\sim 10^{34} \text{ erg s}^{-1}$) is about the same as that of the Crab Pulsar (see Table 2), taking into account the larger distance to the LMC (55 compared with 2 kpc) and the smaller visual extinction (A_V) toward the LMC (0.6 compared with 1.6 mag (ref. 6)). (Here we have used a 0.13 mag colour excess in the LMC from Savage *et al.*⁷ and apply an extra 0.07 mag due to the Milky Way foreground, as suggested by R. P. Kirshner (personal communication), along with his estimated probable error of 0.05 mag.) Our rough estimate shows the pulsar to be significantly red when compared with the Crab Pulsar (see Table 2), the intrinsic colours, $(B - V)_0 \sim 0.7$ and ~ -0.03 respectively^{6,8}. Standard stars were observed in addition to the Crab Pulsar and the central LMC remnant to establish the flux calibrations.

The Crab-like luminosity of the (slower) LMC pulsar (Table 2) may be due in part to beaming effects (a five times larger duty cycle ($\sim 50\%$) than that of the Crab ($\sim 10\%$)), or possibly to a higher efficiency of conversion of mechanical energy into pulsed radiant energy. The nominal magnetic field for this pulsar would be ~ 1.3 times the Crab Pulsar's field (or $\sim 5 \times 10^{12} \text{ G}$); all other parameters are the same for both and use standard arguments⁹. The coincidence of the absolute luminosity of this pulsar and that of the Crab Pulsar, even though the former is 33% slower, argues against emission models predicting optical luminosity as a high power of rotation (see, for example, refs 10, 11), unless beaming effects can be invoked to account for an extra luminosity factor of ~ 20 in the LMC pulsar. Determination of the 'unpulsed' flux from the LMC pulsar will be difficult at best because of the strong central condensation of the nebular synchrotron emission³.

The optical and X-ray pulsed fluxes of the LMC pulsar do not have the same ratio as the corresponding nebular fluxes (which interpolate as, and are both internally consistent with, $\nu^{-0.8}$; refs 2, 3), but instead interpolate as $\nu^{-0.3}$. As the optical and X-ray pulse profiles of the LMC pulsar are identical within

statistics (see also ref. 1), the $\nu^{-0.3}$ relationship is particularly suggestive. This power law would result from the same electron injection spectrum that produces the synchrotron loss-dominated nebular emission spectrum of $\nu^{-0.8}$, except that the electrons producing the optical and X-ray pulses would not have had time to become loss-dominated, but instead have an electron number distribution of $n(E) dE \propto E^{-1.6}$. Such a curve also extrapolates to 0.4 mJy at 400 MHz, a factor of ~ 2 below the present upper limit¹² and perhaps measurable with further effort. Although the spectrum for the pulsed X radiation, corresponding to $\nu^{-0.5}$ from 0.2 to 4 keV (ref. 13), is almost consistent with the interpolated optical-X-ray spectrum, the estimated optical spectrum from B to V , corresponding to $\nu^{-2.8(1.4)}$, is not. The nebular and pulsed fluxes of the Crab also do not each follow a single power law; instead the pattern is much more complex. Although a $\nu^{-0.9}$ interpolation fits the nebular spectrum well from optical to γ -ray energies, a slope change occurs in the corresponding pulsar spectrum and other slope changes occur in both the nebular and pulsar spectra at longer wavelengths⁹.

Because of timing uncertainties, we could not connect phase reliably between the five nights of data. No satisfactory cycle number assignment was found that agreed with the mean $\partial P/\partial t$ value (p -period) for 1979-84 (see also ref. 1) of $41.395 \text{ ns day}^{-1}$. The mean frequency quoted in Table 1 is consistent with the mean of the individual frequencies, but also can be used to count the cycles between runs 2 and 4, and 1 and 5. However,

Table 2 Pulsed fluxes of PSR0540-693

Measured parameters		
$(B + V)/2$ (4-m)		22.70(0.07)
$(B + V)/2$ (1.5-m)		22.34(0.07) + (0.39(0.10))(B - V)*
	Magnitude	Flux density
Derived parameters		
$(B - V)$		0.89(0.35)
B		23.15(0.20)
V		22.26(0.20)
Correcting for absorption, $E(B - V) = 0.20(0.05)$		
$(B - V)_0$		0.69(0.35)
B_0		22.35(0.28)
V_0		21.66(0.25)
L_{B_0}		0.5 $L_\odot$
L_{V_0}		0.7 $L_\odot$
		0.8 $L_{B \text{ Crab}}$
		1.6 $L_{V \text{ Crab}}$

* This term is due to the red response of the GaAs photocathode relative to the S20 extended and was estimated by measurements of standard stars and the Crab Pulsar.

the resulting $\partial P/\partial t$ is 45.9 (0.3) ns day⁻¹ and is inconsistent with the nominal value of 41.4 ns day⁻¹. If this behaviour is confirmed by future observations, then PSR0540-693 would have to be subject to starquakes as large as those found in the Vela Pulsar¹⁴, namely $\Delta f/f \sim 10^{-6}$, where f is the pulse repetition frequency. In this situation, any hope of measuring the braking index, n , in $\partial f/\partial t \propto -f^n$, would be lost. An index of three corresponds to magnetic dipole braking; the Crab Pulsar has an index of 2.5 (ref. 15). However, we must defer judgement on this matter until more-accurately-timed data become available.

In conclusion, the broad pulse profile and the intrinsically red colour render the LMC pulsar distinct from the Crab Pulsar. The relatively high pulsed optical luminosity of PSR0540-693 will facilitate measurements of polarization and colours and, consequently, the refinement of models of pulsed emission. The interpolated $\nu^{-0.3}$ pulsed optical-X-ray spectrum suggests a simplifying assumption, regarding the electron number distribution, which may be valid for such models and which can be tested by slightly more sensitive pulsation limits in radio frequency bands.

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1. Seward, F. D., Harnden, F. R. & Helfand, D. J. *Astrophys. J. Lett.* (in the press).
2. Clark, D. H. *et al. Astrophys. J.* **255**, 440-446 (1982).
3. Chanan, G. A., Helfand, D. J. & Reynolds, S. P. *Astrophys. J. Lett.* (in the press).
4. Middleditch, J. *et al. Nature* **306**, 163-164 (1983).
5. Middleditch, J., Kristian, J. *Astrophys. J.* **279**, 157-161 (1984).
6. Miller, J. S. *Astrophys. J. Lett.* **180**, L83-L87 (1973).
7. Savage, B. D., Fitzpatrick, E. L., Cassinelli, J. P. & Ebbets, D. C. *Astrophys. J.* **273**, 597-623 (1983).
8. Kristian, J., Visvanathan, N., Westphal, J. A. & Snellen, G. H. *Astrophys. J.* **162**, 475-483 (1970).
9. Manchester, R. N. & Taylor, J. H. *Pulsars* (Freeman, San Francisco, 1977).
10. Pacini, F. *Astrophys. J. Lett.* **163**, L17-L20 (1971).
11. Pacini, F. & Salvati, M. *Astrophys. J.* **274**, 369-371 (1983).
12. Manchester, R. N. *Proc. Workshop on Birth and Evolution of Neutron Stars* No. 8 (eds Reynolds, S. P. & Stinebring, D. R.) (National Radio Astronomy Observatory Publications, Greenbank, West Virginia, 1984).
13. Seward, F. D. *Proc. Workshop on the Crab Nebula and Related Supernova Remnants* (eds Kafatos, M. C. & Henry, R. C. B.) (Cambridge University Press, 1984).
14. Downs, G. S. *Astrophys. J.* **249**, 687-697 (1981).
15. Groth, E. J. *Astrophys. J. Suppl.* **29**, 453-465 (1975).

A shadowed flow in the stem of the Crab nebula?

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The faint radio¹ and emission line² 'jet' outward from the northern boundary of the Crab nebula (here called the 'stem') appears as a neat right cylinder (ref. 3 and R. Fesen, personal communication), which is lengthening and expanding. Here we model the glowing cylinder as the convected margin of a gas cloud that accidentally cast its shadow across the nearly ballistic flow of the stellar

envelope ejected in the supernova explosion. We show that this model is consistent with known data on the stem and that it accounts for the strikingly regular geometrical features in a natural way. In contrast, flow instability models²⁻⁶, although in many ways appealing, do not easily result in so neat a cylinder.

Recent measurements have greatly clarified the nature of the stem. It is moving away from Earth at a speed comparable with that of the Crab nebula itself^{2,3}. Proper motion has been observed in one of the knots of the stem, implying a tentative value of 4,000 km s⁻¹ for the motion of the stem outward from the nebula (ref. 3 and R. Fesen, personal communication). These two observations combine to say that the stem lies within 10° of the plane of the sky with actual dimensions very close to the projected ones, 0.5-pc width and 1.5-pc length (assuming a 2-kpc distance for the nebula). Line splittings have been found which are most readily explained as the expansion of a cylindrical structure at a speed perhaps as high as 360 km s⁻¹ (refs 3 and 4). The composition of the stem is not that of the average interior nebula filament, the stem being relatively helium-poor³, although the optical filaments within the northern nebular edge are closer to stem composition than is the nebula as a whole⁷. There appears to be a gap in the complex filamentary structure near the base of the stem^{3,8}; the projection of the stem axis back into the nebula definitely misses the explosion centre.

Many models of the stem, including ours, assume as a basis Chevalier's model⁹ of the Crab nebula as the outcome of a Type II massive supernova explosion. In particular, this model states that the ejected stellar envelope should be found in a 'halo' outside the filamentary stellar core remnant, moving outward with a maximum speed in excess of 5,000 km s⁻¹. A faint halo seemed to be confirmed by the observations of Murdin and Clark¹⁰. Although doubt has been cast on those results⁸, it is certain that the halo should be both difficult to detect and yet the seat of the mass needed to make a Type II supernova model work⁹. More sensitive observations (optical and radio) will be very useful in sorting out the various proposed stem models.

The stem motion seems to rule out the 'star trail' model^{3,11}; the other motions argue against any model involving ejection of material directly from the pulsar or the explosion centre. Apart from the present model, this leaves instability models as the only probable contenders. In our model, the stem is produced by the entrainment of cloud material at its interface with the highly supersonic flow of the ejected stellar envelope. The high Mach number ($M \gg 10$) implies that there will be little flow recvergence behind the shadowing cloud. On the other hand, the simplest version of this model would produce a cone rather than a true cylinder of gas, so the cloud must have been moving south-east to produce nested cones of flow. The remarkably straight western edge of the stem (not its central axis) points directly to the explosion centre, as we would expect for one edge of the shadow, whereas the eastern edge is less even, consistent with the idea that it represents the leading edge of a succession of cones, unlike the single initial formation on the opposite side.

To make the model more quantitative, suppose the cloud was initially located about two-thirds of the way from the explosion centre to the current edge of the filaments. To contribute any significant mass to the nebula, the envelope must have a density of ~ 10 cm⁻³ or more at the time it reaches the cloud. This means that the cloud density must be 100 cm⁻³ so that it will not be swept away too fast. There are many such condensations in the galactic plane, but they will be rare at the 200-pc distance of the Crab from the plane. The low probability of such a cloud, although in one way a difficulty, supports this model, as it makes it reasonable that there should be only one stem. The transverse motion of the cloud will have to be ~ 100 km s⁻¹; it would be slower if the cloud were closer in, but then the cloud would have to be more dense.

Given these parameters, the process we envision has three stages. In the first, the cloud is probably completely ionized by the radiation from the supernova in the approximately 200-300 yr before the cloud is struck by the flowing gas (compare

with the ionization of the star trail in the Blandford *et al.*¹¹ model). Over the next 150 yr or so, the cloud is compressed (primarily along the direction of the supersonic flow at high Mach number), and its surfaces stripped of gas. The edge interaction results both from initial irregularities in the cloud, and from instabilities (say of Rayleigh–Taylor type) that develop along the cloud–ejecta interface. The speed of the compression is given roughly by the square root of the density ratio, or $\leq 2,000 \text{ km s}^{-1}$, implying a compression of the entire 0.3-pc cloud in 150 yr. This is enough time to produce the visible stem.

In the final phase of stem development, the stripped fragments of the cloud are accelerated to the speed of the ejecta, and the cloud as a whole is accelerated and dispersed; any last remnants of the cloud are certainly dispersed and/or obscured by the advancing filaments. The stem gas^{3,4} will be heated in the acceleration process. Although the details of this process are difficult to calculate, the initial relatively high density of the emitting condensations may help ensure that the convected gas is not too hot to give line emission at present³. In the shadow model, the radio stem would be produced by electrons accelerated in the shocks and turbulence produced during the cloud–ejecta interactions, and subsequently convected with the flow.

As the flow past the cloud is conical, we expect the optical line to show a ‘velocity ellipse’ with components of motion along the line-of-sight, as observed³. The outward stem motion is about equal to that of the slower ejecta around it; the model predicts a decrease from $\sim 2,500 \text{ km s}^{-1}$ at the distal end to $< 2,000 \text{ km s}^{-1}$ at the base⁸. (The initial estimate of the observed proper motion is higher.) The half-apex angle of the cone is about 1/12 (one-half the stem width divided by the distance from the explosion centre to the far end of the stem). That implies a stem expansion speed of $\sim 200 \text{ km s}^{-1}$. This value is much less than that given by Shull *et al.*³, but it is in quite good agreement with the shift measured in the Doppler centre of the line profile. The rest of the line width can be readily explained by turbulent spreading of the profile. The base of the stem shows much-broadened lines, expected from the overlapping cones of flow and the mature turbulence in the final stages of the convective process. No other theory accounts for the alignment of the western stem edge with the explosion centre, or for any, other than very rough, quantitative details of the kinematics. (The motions are used only as input parameters in most models.)

Perhaps the most appealing qualitative model is one that supposes the stem to consist of local filamentary material that was blown out through the advancing surface of the nebula by the pressure of the relativistic plasma within^{2,5}. This model can dispense with a halo and it quite naturally explains why the stem radio emission shows up as a slightly weaker extension of the nebular emission. Magnetic tension is probably needed to keep the stem walls from rapidly diverging on this view (see ref. 3), but even with the necessary fields the analogy of an elastic membrane with one weak point implies not a straight narrow structure, but something more of a puff or bubble. How is the roughly isotropic relativistic gas pressure, many orders of magnitude higher than the ram pressure, able to produce both rapid outward motion and a slow transverse motion? Finally, if the sharp bend at the stem–filament boundary is the result of, say, mass loading of field lines by filaments at the base, then we would expect little motion there, as opposed to the cylindrical expansion observed.

One way around some of these difficulties is suggested by the more quantitative model of Shull *et al.*³, which suggests a non-relativistic plasma jet, with ram pressure much higher than thermal pressure, as the source of the stem. That model does not account for the continuity of radio intensity into the stem. It seems implausible also that compression of the halo gas can produce the optical emission, as the required compression by more than a factor of 10 within the weakly radiative shock that generates the density enhancement is unlikely. Even if we accept filament material as the source of emitting stem matter, there is no obvious concentrated energy source to produce a directed flow. The magnetic energy available from the very weak filaments

at the outer edge of the nebula seems much too small to produce such a jet. There is no obvious coherent energy source within the tangled web of filaments. All jet theories share this weakness.

Further observations are needed. If sensitive observations do not detect a halo, then the shadow model and models relying on halo compression may be rejected. If the outward motion of the stem is shown to be much faster than $2,500 \text{ km s}^{-1}$, the shadow model is unlikely. A steeper radio spectral index for the cloud than for the nebula as a whole (which has a flatter spectrum than that expected from shock acceleration) would favour our model, as would any evidence for a stem composition implying a mixture of some interstellar medium. Of course, the discovery of other stems in this or in other supernova remnants would provide more opportunities for tests, but as yet the stem is unique.

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1. Velusamy, T. *Nature* **308**, 251–252 (1984).
2. Gull, T. R. & Fesen, R. A. *Astrophys. J. Lett.* **260**, L75–L78 (1982).
3. Shull, P. *et al. Astrophys. J. Lett.* (in the press).
4. Chevalier, R. A. & Gull, T. R. *Astrophys. J.* **200**, 399–401 (1975).
5. Bychkov, K. V. *Soviet Astr.* **18**, 420–425 (1975).
6. Kundt, W. *Astr. Astrophys.* **121**, L15–L18 (1983).
7. Trimble, V. *Nature* **313**, 96 (1985).
8. Clark, D. H. & Murdin, P. *et al. Mon. Not. R. astr. Soc.* **204**, 415–431 (1983).
9. Chevalier, R. A. in *Supernovae* (ed. Schramm, D.) (Reidel, Boston, 1977).
10. Murdin, P. & Clark, D. H. *Nature* **294**, 543–544 (1981).
11. Blandford, R. D., Kennel, C. F., McKee, C. F. & Ostriker, J. P. *Nature* **301**, 586–587 (1983).

X-ray morphology of the Crab nebula

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The Crab nebula is so far the only object where the direct interaction between a pulsar and the surrounding remnants of the supernova can be studied. Here we present an X-ray image of the Crab nebula taken with the Einstein Observatory High Resolution Imager (HRI) and deconvolved using Fourier techniques. The picture uncovers for the first time spatial structures that further refine the ideas about the energy injection mechanism and the electron propagation process in the nebula.

The lifetime of the synchrotron-radiating relativistic electrons in the Crab nebula is so short that these cannot be ejected in the supernova explosion seen in AD 1054 and have to be replenished continuously. This has stimulated several investigations of the injection and propagation of electrons in the nebula^{1–3}. Generally, the shrinking size of the nebula with increasing energies is explained by the decreasing lifetime of the radiating electrons; also, as the dimensions of the ‘injector’ (the pulsar) are negligibly small, the theories predict a point source at high X-ray energies centred on the pulsar. However, from the first lunar occultation observation of the Crab nebula⁴, there were indications of an offset of the emission centroid from the pulsar in a northwesterly direction. This was later confirmed by a series of occultation experiments in 1974^{5,6}, which also demonstrated that even at energies $> 20 \text{ keV}$, the X-ray source had a finite size of $\geq 20 \text{ arc s}$.

Although the Einstein telescope was the first imaging instrument of sufficient spatial resolution to provide X-ray images of the central region of the Crab nebula, in the published pictures⁷ the information about size and shape of the emission region is masked by the convolution effect with the HRI’s point spread function. Figure 1 shows the picture we obtained for the Crab nebula after processing the original data, taken during the off-

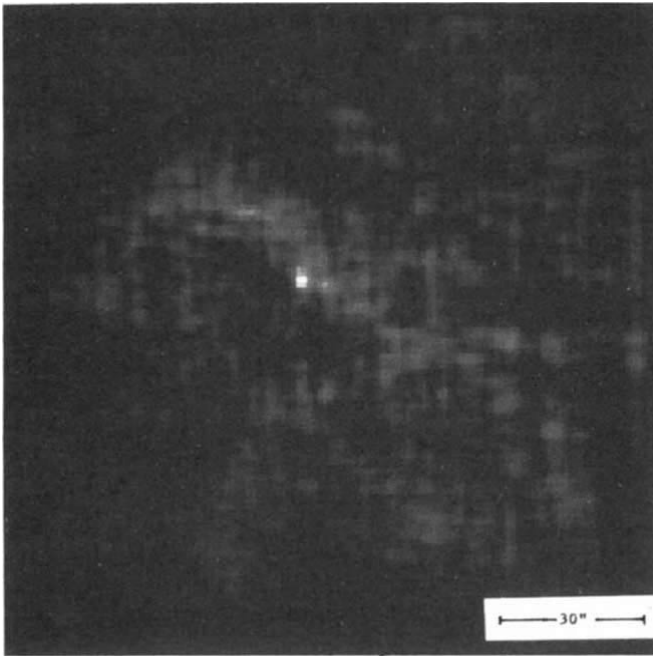


Fig. 1 The X-ray emission region of the Crab nebula obtained by processing the off-pulse Einstein HRI data.

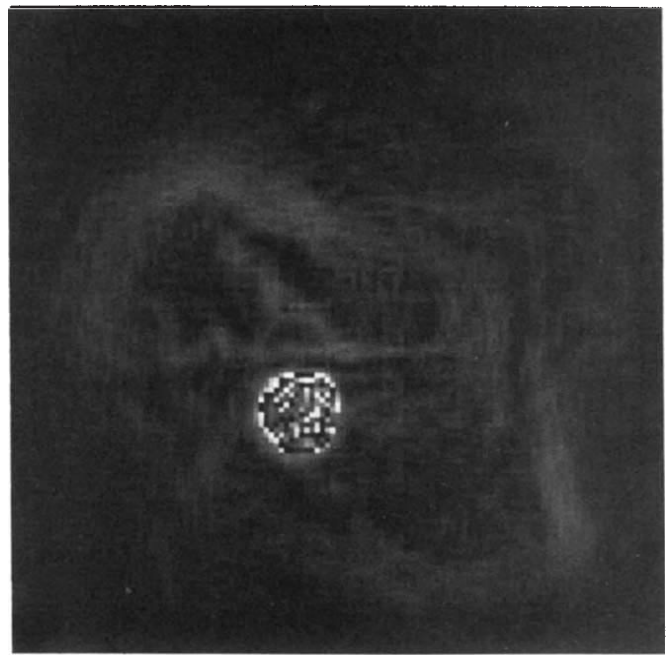


Fig. 2 The Einstein HRI data of the Crab nebula in a gradient representation.

pulse phases of the pulsar. The pixel size is $1 \text{ arc s} \times 1 \text{ arc s}$. The data contain roughly $15 \times 10^3 \text{ s}$ of net observation time. For the deconvolution we used a Fourier-transform technique⁸ with the on-axis full-range point-spread function at $8.3 \text{ \AA}$ (ref. 9). The data were first smoothed with a gaussian filter and then Fourier transformed in two dimensions, applying an appropriate data window to correct for the finite extent of the picture¹⁰. The frequency spectrum was then divided by the Fourier transform of the point-spread function and transformed back into the spatial domain, giving the intensity distribution of Fig. 1.

Although there is substantial noise in the picture, a ring-like structure of the emission region is clearly visible including the central hole, known from the optical image. The dark unstructured parts of the picture contain signals that are below $\sim 10\%$ of the maximum intensity. The pronounced enhancement of emission in the north-west region of the pulsar is a factor of ~ 3 stronger than the average emission, which explains the offset found in the earlier measurements. The brightest point in the picture is consistent with the position of the 'star' seen in the optical picture $\sim 25 \text{ arc s}$ north of the pulsar.

The overall geometrical configuration as well as the size of the inner and outer elliptical contours and the ratio of the major to minor axes of the ring-like emission structure agree closely with the predictions of a model proposed by Aschenbach and Brinkmann¹¹ in which the electrons are accelerated and then transported outwards, radiationlessly, in the strong electromagnetic wave of the pulsar. When the ram pressure of this relativistic wind equals the thermal pressure of the surrounding nebula, a shock forms, producing an amplification of the magnetic field and a randomization of the directed plasma motion¹². Only from this radial distance and beyond do the electrons lose their energy by synchrotron radiation, thus leaving an emission hole around the pulsar. Taking for the pulsar the field configuration of an oblique rotator, the acceleration and outflow of the electrons are most efficient near the rotational equatorial plane¹³, producing a toroid-like structure for the emission region. The height of this toroid is determined by comparing the lifetime of the radiating electrons with their effective radial propagation time. The brightness asymmetry in the optical picture near the pulsar and the X-ray offset was explained by an enhancement of the magnetic field strength in the northwestern parts of the nebula. As the proper motion of the pulsar is in that direction¹⁴, it was

proposed that the necessary field amplification is a compressional effect of the pulsar running into the surrounding medium.

In 1975 this model was fitted to the available optical picture of the central region of the Crab and some rough estimates about the expected size and brightness distribution of the X-ray emitting region were made. The predicted values are in good qualitative agreement with the Einstein picture. A detailed analysis of the brightness distribution is now under way (T. Bork, unpublished thesis).

In addition to the toroidal shape, the soft X-ray as well as the optical picture exhibits the so-called 'anvil'. In our model, this structure, as well as the wisps, is correlated with plasma ejection processes from the (rotational) polar regions of the pulsar, although the structures are on large scales not co-aligned with the rotational axis of the neutron star. The bending of the jet may be the result of interaction of the outstreaming plasma with the surrounding nebular matter, by processes similar to those discussed in the context of curved jets of radio galaxies¹⁵. The horizontal linear feature, visible in Fig. 1, is probably an artefact. It masks exactly the position of the wisps, which in some models are regarded as the centres of the activity of the nebula. In those models, the wisps are produced by electromagnetic instabilities in the wind-wave system. Again the relativistic electrons are transported without radiation losses to this region, where the instability suddenly releases the wave energy into proper modes of the wind plasma and radiation ensues by the Compton-synchrotron process¹⁶. Although there is some X-ray flux enhancement in this part of the picture, we cannot attribute it definitely to the wisps' activity, nor can we reject this hypothesis.

The total Einstein HRI data from the Crab consist of more than $45 \times 10^3 \text{ s}$ of useful data. Unfortunately, they could not be used to improve the statistics, as our technique is very sensitive to the large-scale systematic erroneous structures existing in the convolved image. Figure 2 shows the whole data in a gradient representation, an ideal method for edge enhancement, in terms of the quantity $P_{gr}(x, y) = \sqrt{(\partial P(x, y)/\partial x)^2 + (\partial P(x, y)/\partial y)^2}$, where $P(x, y)$ is the intensity in the image pixel with coordinates (x, y) . A comparison of this representation with the Fourier deconvolved data (Fig. 1) demonstrates the reality of the ring structure. An imperfect correction for distortions introduced by the HRI detector electronics, inherent in the original data¹⁷, is

clearly visible as a bar-like structure just north of the pulsar. These spatially extended oriented features degrade the Fourier spectrum so that the quality of the deconvolved pictures can be little improved beyond that of Fig. 1. We hope that in a reconstruction of the pictures this problem can be overcome.

High-energy X-ray data (21–63 keV) taken with a rotation modulation collimator balloon experiment¹⁸ show that, with increasing energy, the emission becomes progressively more concentrated to the northwestern region of the pulsar. This is a strong argument in favour of the Aschenbach and Brinkmann model, where the X-ray morphology mirrors the toroidal emission volume outside the central hole.

Although in the original model the geometrical configuration of the emitting region is at least qualitatively correct, a comparison of the available hard and soft X-ray data with numerically simulated brightness distributions indicates that the underlying particle injection and transportation mechanism cannot be as simple as anticipated. The spatial variations of the intensity ratios at different X-ray energies and the well-known overall power law spectrum of the Crab nebula are hard to explain by a model in which electrons are injected into the nebula and can then evolve freely—even if an ‘unorthodox’ magnetic field configuration is chosen. It is more probable that a more complex mechanism including particle re-acceleration (such as that proposed by Kennel *et al.*¹⁹) is involved or that the energy injection mechanism is quite different²⁰.

For a more detailed study of these possibilities, X-ray pictures with high spectral and spatial resolution are required. A further advance can be expected from the data collected recently by the Exosat satellite, which are now being analysed.

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1. Wilson, A. *Mon. Not. R. astr. Soc.* **160**, 355–371 (1972).
2. Gratton, L. *Astrophys. Space Sci.* **16**, 81–100 (1972).
3. Cocke, W. J. *Astrophys. J.* **202**, 773–781 (1975).
4. Bowyer, C. S., Byram, E. T., Chubb, T. A. & Friedman, H. *Science* **146**, 912–917 (1964).
5. Staubert, R. *et al. Astrophys. J. Lett.* **201**, L15–L19 (1975).
6. Ku, W., Kestenbaum, H. L., Novick, R. & Wolff, R. S. *Astrophys. J.* **204**, L77–L81 (1976).
7. Seward, F. D. in *Proc. Nato Advanced Study Institute ‘Supernovae’* (eds Rees, M. J. & Stoneham, R. J.) (Reidel, Dordrecht, 1981).
8. Moik, J. G. *Digital Processing of Remotely Sensed Images* (NASA SP-431, 1980).
9. Zombeck, M. V., Austin, G. K. & Torgerson, D. T. *Smithsonian Astrophysical Observatory spec. Rep. SAO-AXAF-80-003* (1980).
10. Brault, J. W. & White, O. R. *Astr. Astrophys.* **13**, 169–189 (1971).
11. Aschenbach, B. & Brinkmann, W. *Astr. Astrophys.* **41**, 147–151 (1975).
12. Rees, M. J. & Gunn, J. E. *Mon. Not. R. astr. Soc.* **167**, 1–12 (1974).
13. Ferrari, A. & Trussani, E. *Astrophys. Space Sci.* **33**, 111–126 (1975).
14. Trimble, V. *IAU Symp.* **46**, 12–21 (1972).
15. Fomalont, E. B. in *Astrophysical Jets* (eds Ferrari, A. & Pacholzyk, A. G.) (Reidel, Dordrecht, 1983).
16. Bendford, G., Bodo, G. & Ferrari, A. *Astr. Astrophys.* **70**, 815–820 (1978).
17. Harnden, F. R. Jr. & Seward, *Astrophys. J.* **283**, 279–285 (1984).
18. Makishima, K. *et al. Space Sci. Rev.* **30**, 259–262 (1981).
19. Kennel, C. F. & Coroniti, F. V. *Astrophys. J.* **283**, 694–709 (1984).
20. Michel, F. C. in *The Crab Nebula and Related Supernova Remnants* (ed. Kafatos, M.) (Cambridge University Press, 1985).

Experiment and theory of ‘transparent’ metal films

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We report here the optical properties of near-transparent porous platinum films supported on p-type indium phosphide (InP). The deposition of small quantities of catalytically active metals on p-InP increases photoelectrochemical solar-to-hydrogen conversion efficiency by providing a pathway for photogenerated electrons to reduce efficiently protons in solution¹. Surprisingly, efficiencies increase slowly even after fairly thick metallic layers have been deposited. These results imply that in certain conditions the metal overlayer is effectively transparent to the incident light, or even promotes the coupling of incident radiation into the bulk of the semiconductor². We present here both experimental and theoretical

evidence to show that these effects can be understood in terms of microstructure: when the metal films are sufficiently porous and built up from particles smaller than the wavelength of the transmitted light, the photon fields are screened out of the metal phase and are forced into the void structure. This increases the effective refractive index of the layer over that of the ambient and provides a better match with the substrate, while incurring negligible absorption loss.

Photoelectrodeposition onto a p-type semiconductor substrate was used to form the metal films for optical study. This method offers several advantages. Light of energy greater than the band-gap is absorbed within the semiconductor and generates charge carriers. At sufficiently negative applied potentials, photogenerated conduction band electrons migrate to the surface and are available to reduce species in the contacting solution. In this way, metals can be plated from solutions of their salts. Deposition is turned on or off simply by turning on or off the incident light. Metal nucleation and growth rates, which determine the form and microstructure of the deposit, can be controlled through solution composition, mass transport conditions, applied potential and incident light intensity. In the present study, we used low metal ion concentrations, no complexing or surface active species, low rates of mass transport, and applied potentials and light intensities such that the concentration of photogenerated electrons greatly exceeded the concentration of Pt(IV) at the semiconductor–electrolyte interface. These conditions favour the deposition of a metal with an open microstructure and result in improved transparency. The plating solution was halide-free, of composition 1.00 mol dm^{−3} HClO₄ + 1.19 × 10^{−5} mol dm^{−3} Pt(IV) and was transparent from 1.5 to 6.0 eV. This allowed the deposition process to be monitored directly by spectroscopic ellipsometry.

Through efficient faradaic reactions, the semiconductor can be used as a photodetector to monitor directly the flux of photons transmitted through the metal layer. In our experiment, the collection efficiency of photogenerated electrons in the faradaic mode is given by

$$N = \phi N_F(E) \quad (1)$$

where ϕ is the optical coupling efficiency (ratio of penetrating to incident photons), and $N_F(E)$ is the potential-dependent faradaic collection efficiency³, which takes into account the competition between charge transfer to solution species and carrier recombination within the semiconductor. Here, we used Pt-catalysed hydrogen evolution as our actinometer, because it is nearly 100% efficient^{1,2} for this system. Because $N_F(E)$ can approach one at sufficiently negative applied potentials, N is a good measure of ϕ in those conditions.

Ellipsometric spectra were obtained using a rotating-analyser instrument described elsewhere⁴. The sample cell was fitted with quartz windows to allow complex reflectance ratios to be measured *in situ* over the entire accessible 1.5–6.0 eV photon energy range, to ensure accurate microstructural analysis. *In situ* measurement also allowed film evolution to be followed with the metal film in its unperturbed state, the only variable being the film thickness, d , during an experiment. We define the complex pseudodielectric function $\langle \tilde{\epsilon} \rangle = \langle \epsilon_1 \rangle + i \langle \epsilon_2 \rangle$ to be that quantity obtained when the measured complex reflectance ratio is transformed by the so-called two-phase (substrate–ambient) model into the bulk dielectric function of a hypothetical sample with no overlayers present. If overlayers are present, $\langle \tilde{\epsilon} \rangle$ is an average over both overlayer and bulk values.

The InP surface was prepared using previously described methods⁵, and its quality was verified by ellipsometric measurements before plating. Comparison of the initial data with the InP dielectric function data base⁶ showed that the surface had <1.5 Å microscopic roughness, but also showed that the reported refractive index dispersion curve for H₂O (ref. 7) had to be increased by 1.6% across the investigated spectral range to account for the extra polarizability of the dissolved perchlorate ions. Plating was performed at 0.236 V (SHE), spectra were recorded at 0.436 V and collection efficiencies determined at

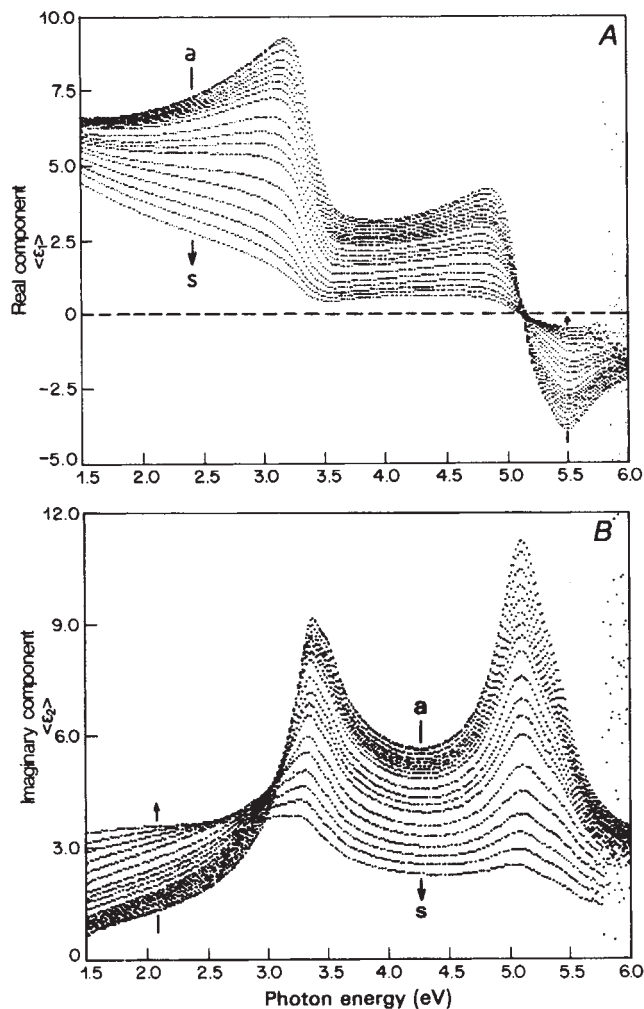


Fig. 1 Evolution of the complex pseudodielectric functions ($\bar{\epsilon}$) for the p-InP(Pt)/1.00 mol dm⁻³ HClO₄ system during the course of a deposition experiment. The $\langle \epsilon_1 \rangle$ (Fig. 1A) and $\langle \epsilon_2 \rangle$ (Fig. 1B) components of ($\bar{\epsilon}$) were calculated from ellipsometric parameters measured during the experiment. The initial spectrum (a) was for the Pt-free (100)p-InP surface in the plating solution. The arrows indicate the changes observed with increasing Pt loading. The final spectrum (spectra) yielded the following best-fit parameters when subjected to LSR analysis as described in the text: $d = (277.3 \pm 12.6) \text{ \AA}$, $f_v = (0.838 \pm 0.019)$, $\kappa = (1.79 \pm 0.17)$. Spectra b-s were obtained, from $d = (2.6 \pm 0.8) \text{ \AA}$ (spectrum b), at approximately equal intervals in log d . The features of the InP substrate spectrum were still visible even for the thickest metal layer, graphically illustrating the transparency of the overlying metal film.

-0.364 V. Normal incidence illumination of the sample was at $888 \pm 21 \text{ nm}$ and an incident power density of 0.43 mW cm^{-2} . The results of the ellipsometric measurements are shown in pseudodielectric function ($\langle \bar{\epsilon} \rangle$) form in Fig. 1, and the photoelectrochemical collection efficiencies as curve b in Fig. 2. Even for the thickest metal film the substrate spectral features can be clearly seen, giving direct evidence of the essential transparency of the overlayer.

The ellipsometric data were analysed using standard procedures⁸. The sample configuration was represented by the three-phase (substrate-film-ambient) model. The ($\bar{\epsilon}$) spectrum for the bare immersed InP surface was used as the substrate reference and the corrected dispersion curve of H₂O as that of the ambient. The metal film, being macroscopically homogeneous but microscopically heterogeneous, was described by an effective medium mix of the dielectric functions of Pt and the ambient fluid. Little difference was observed between the Bruggeman⁹ (random or aggregate) and the Maxwell Garnett cermet¹⁰ (coated-sphere) microstructural models, but the former was used on the basis

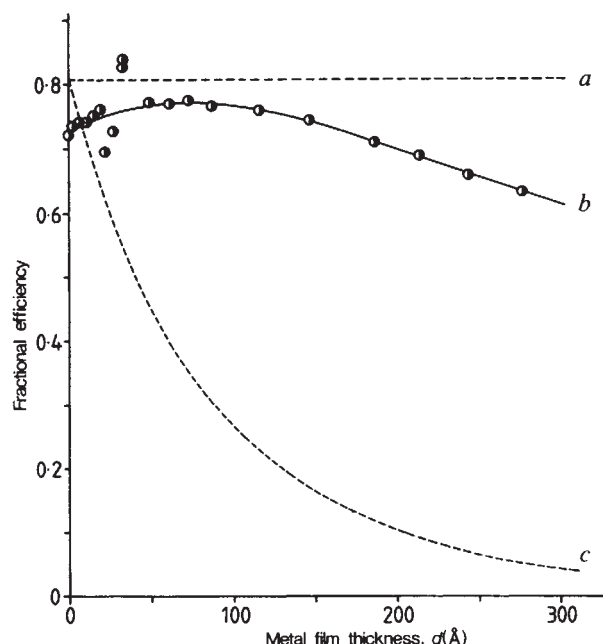


Fig. 2 Curve a shows the optical coupling efficiency ϕ_{827} , describing the penetration of 827 nm (1.50 eV) light through to the InP substrate, as a function of platinum film thickness. It was calculated from the best-fit values of d , f_v and κ obtained from the spectra of a deposition experiment. The $\langle \epsilon_1 \rangle$ (A) and $\langle \epsilon_2 \rangle$ (B) components of ($\bar{\epsilon}$) were calculated from ellipsometric parameters measured during the experiment. The initial spectrum (a) was for the Pt-free (100)p-InP surface in the plating solution. The arrows indicate the changes observed with increasing Pt loading. The final spectrum (spectra) yielded the following best-fit parameters when subjected to LSR analysis as described in the text: $d = (277.3 \pm 12.6) \text{ \AA}$, $f_v = (0.838 \pm 0.019)$, $\kappa = (1.79 \pm 0.17)$. Spectra b-s were obtained, from $d = (2.6 \pm 0.8) \text{ \AA}$ (spectrum b), at approximately equal intervals in log d . The features of the InP substrate spectrum were still visible even for the thickest metal layer, graphically illustrating the transparency of the overlying metal film.

of the connectivity evidence provided by the observed electrocatalytic activity of the metal layer and because of its method of preparation. The Maxwell Garnett honeycomb¹⁰ (contiguous metal skeleton) model was completely inconsistent with the data, and could be rejected.

The Bruggeman effective medium approximation used here for the metal film is

$$0 = f_m \left(\frac{\tilde{\epsilon}_m - \tilde{\epsilon}_f}{\tilde{\epsilon}_m + \kappa \tilde{\epsilon}_f} \right) + f_v \left(\frac{\tilde{\epsilon}_v - \tilde{\epsilon}_f}{\tilde{\epsilon}_v + \kappa \tilde{\epsilon}_f} \right) \quad (2)$$

where f_x is the volume fraction of constituent x and $\tilde{\epsilon}_x$ its complex dielectric function, $\tilde{\epsilon}_f$ is the estimated effective dielectric function of the film, κ is a screening parameter and m denotes metal and v void. Least-squares regression (LSR) was used to determine the values of the layer thickness d , the void (ambient fluid) fraction f_v and the screening factor κ of the metal film that gave the best representation of the dielectric data. For ideal isotropic screening in one, two or three dimensions, κ assumes the value 0, 1 or 2 respectively. The model gives a precise description of both real and imaginary parts of all spectra shown in Fig. 1, thereby demonstrating its completeness and accuracy. The best-fit values of the void fraction f_v obtained from the spectra of Fig. 1 were all high, ranging from ~0.6 for the thinnest metal film to ~0.85 for the thickest films. Therefore, the films all had a very open microstructure.

The transmittance of the metal film based on the ellipsometrically-determined parameters is shown as curve a in Fig. 2 and the photoelectrochemical collection efficiency as curve b in Fig. 2. The film is seen to be transparent even up to thicknesses approaching 300 $\text{\AA}$. The fair agreement between the collection efficiencies and the computed transmittances (compare with

equation (1)) shows that the same effective medium approach can be used to understand the physical origin of the transparency of the metal film. For simplicity, we expand the calculated transmittance to first order in the relative thickness d/λ and the metal fraction $f_m = 1 - f_v$, finding for the fractional intensity entering the substrate

$$(I_t/I_0) = \phi \approx \frac{4n_a \operatorname{Re}(\tilde{n}_s)}{|\tilde{n}_s + n_a|^2} \left[1 - \frac{4\pi d}{\lambda} \operatorname{Im} \left(\frac{\tilde{\epsilon}_t + \tilde{n}_s n_a}{\tilde{n}_s + n_a} \right) \right] \quad (3a)$$

$$\approx \frac{4n_a n_{sr}}{|\tilde{n}_s + n_a|^2} \left\{ 1 + \frac{4\pi d \epsilon_a (1 + \kappa) f_m}{\lambda |\tilde{n}_s + n_a|^2} \times \left[n_{si} - \frac{\epsilon_a (1 + \kappa)}{|\tilde{\epsilon}_m|^2} \operatorname{Im}((\tilde{n}_s + n_a) \tilde{\epsilon}_m) \right] \right\} \quad (3b)$$

where $\tilde{\epsilon}_s = \tilde{n}_s^2 = (n_{sr} + in_{si})^2$, $\tilde{\epsilon}_t$, $\tilde{\epsilon}_m$ and ϵ_a are the dielectric functions of substrate, film, metal and ambient respectively and Re and Im are real and imaginary projections. Equation (3b) follows from equation (3a) for either Bruggeman or cermet-Maxwell Garnett expressions because it is of first order in f_m .

The substrate and ambient being defined, and $\tilde{\epsilon}_s$ and ϵ_a therefore fixed, equation (3b) shows that maximum transmittance through the film can be achieved by minimizing the negative term. The proper approach is to minimize the screening factor κ and maximize $|\tilde{\epsilon}_m|$, that is, to maximize the effective screening and force as completely as possible the photon field into the intervening spaces between the metal particles. To the extent that this can be accomplished, the remaining first-order term, of positive sign, shows that the transmittance will actually increase with increasing metal fraction and film thickness. By compressing the photon fields in this way, the effective refractive index of the fluid in the interparticle regions is increased, thereby providing a better optical impedance match between substrate and ambient. Through numerical calculations using the exact three-phase Fresnel expressions, we have found that a further anti-reflection effect becomes operative as the film approaches quarter-wave thickness, because of multiple reflections within the film. This interference effect, not described in equations (3a and b) can provide additional transmittance. Thus the transparency of the metal films is understood.

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1. Heller, A., Aharon-Shalom, E., Bonner, W. A. & Miller, B. *J. Am. chem. Soc.* **104**, 6942-6948 (1982).
2. Heller, A. *Science* **223**, 1141-1148 (1984).
3. Albery, W. J. & Bartlett, P. N. *J. electrochem. Soc.* **130**(8), 1699-1706 (1983).
4. Aspnes, D. E. & Studna, A. A. *Appl. Opt.* **14**, 220-228 (1975).
5. Aspnes, D. E. & Studna, A. A. *Appl. Phys. Lett.* **39**, 316-318 (1981).
6. Aspnes, D. E. & Studna, A. A. *Phys. Rev.* **B27**, 985-1009 (1983).
7. Hale, G. M. & Querry, M. R. *Appl. Opt.* **12**, 555-563 (1973).
8. Aspnes, D. E., Theeten, J. B. & Hottier, F. *Phys. Rev.* **B20**, 3292-3302 (1979).
9. Bruggeman, D. A. G. *Ann. Phys. (Leipzig)* **24**, 636-657 (1935).
10. Maxwell Garnett, J. C. *Phil. Trans. R. Soc. A* **203**, 385-420 (1904); **205**, 237-288 (1906).

Cathodoluminescence of catalyst crystallites

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Heterogeneous catalysis has played a major role in the development of the chemical industry¹. Metal oxide catalysts, for example, are used in selective oxidation and ammoxidation of hydrocarbons to produce key industrial chemicals. The activity and selectivity of such catalysts are greatly influenced by their microstructural and electronic structure changes under operating conditions. Here we

report the first investigation of the electronic properties of small selected areas of catalyst particles, each only a few micrometers in extent, using a scanning electron microscope technique originally developed for characterizing semiconductor materials. The wavelength of the light emitted by selective electron probe stimulation (cathodoluminescence) can be directly related to the local bandgap energy of the material and the intensity of the signal to the point defect level. Some interpretation is required, but there are minimal specimen preparation requirements and the process is essentially non-destructive so that development sequences can be followed. In favourable cases the important aggregations of point defects can be mapped with respect to other microstructural features. An electronic contribution to promoter mechanisms is suggested.

Certain extended defects, for example, crystallographic shear (CS) defects introduced into some oxide catalysts to accommodate the non-stoichiometry (a result of the loss of lattice oxygen for catalysis) are thought to be relevant for oxygen exchange and catalytic activity in selective oxidation reactions². However, operation without such defects has been established for commercially important Te and Bi molybdate systems under operating conditions using a combination of *in situ* (dynamic) electron microscopy (EM), high resolution EM and analytical EM (AEM)^{3,4}. Direct studies coupled with parallel chemical experiments on some model catalyst systems, for example MoO₃ and V₂O₅, have also indicated that the CS defects in these play only a limited role in the reactions⁵, suggesting that they are a consequence or an accommodation of the changes produced by the catalytic activity (for example, an accommodation of supersaturation of vacancies), rather than the origin of reactivity.

From the studies on model as well as commercial catalyst systems it is clear therefore that in addition to microstructural changes, point defect density and electronic structure can both have a significant influence on the chemical properties and hence on the effectiveness of the material as a catalyst. Scanning electron microscopy (SEM) and probe techniques commonly applied to semiconductor materials^{6,7} have been used to examine this and to supplement information from *in situ* EM, ultra-high resolution structure imaging, conventional AEM and electron probe chemical microanalysis (EPMA)⁸. The initial emphasis has been to use cathodoluminescence, which, unlike electron beam induced conductivity, is a non-contacting technique and does not have any special specimen requirements. It is limited to bandgaps in the optical range (1-5 eV), but this covers many of the materials of interest. Experiments are possible with very small, usually single crystal, particles only a few micrometres in size, and generally very similar to the catalyst samples used in practical applications. The large single crystals required by more conventional methods for determining electronic properties are not usually available for these complex powdered catalyst materials (and with large single crystals it is difficult to obtain the relevant products by reaction under realistic conditions). The data available in the literature⁹ are consequently very incomplete, precluding comparison with theoretical calculations¹⁰ and perhaps resulting in an over-emphasis on structural factors in evaluating catalytic compounds.

Experiments were carried out on materials of interest in catalysis, namely, rutile (TiO₂), mixed Sn/Sb oxides and Bi molybdate systems. The TiO₂ powder was crushed from a boule (National Lead Company, USA)¹¹; the mixed Sn/Sb oxide catalyst powders were prepared by co-precipitation using appropriate amounts of SnCl₄ and Sb/aqueous ammoniacal solution; Bi molybdate powders were prepared by co-precipitating aqueous Bi nitrate and ammonium para-molybdate solutions⁴. The catalyst particles were several micrometres in extent in each case and were dispersed on the usual carbon support films for microscopy. Reduction experiments were performed on the catalysts (using (111) rutile and Sn/Sb oxide crystallites and (001) Bi molybdate), *in situ*, in a high voltage EM (an AEI-EM7 HVEM) fitted with a gas reaction cell⁴, in a controlled H₂ environment and at operating temperatures, with and without the electron beam, to obtain the reaction sequences. Parallel

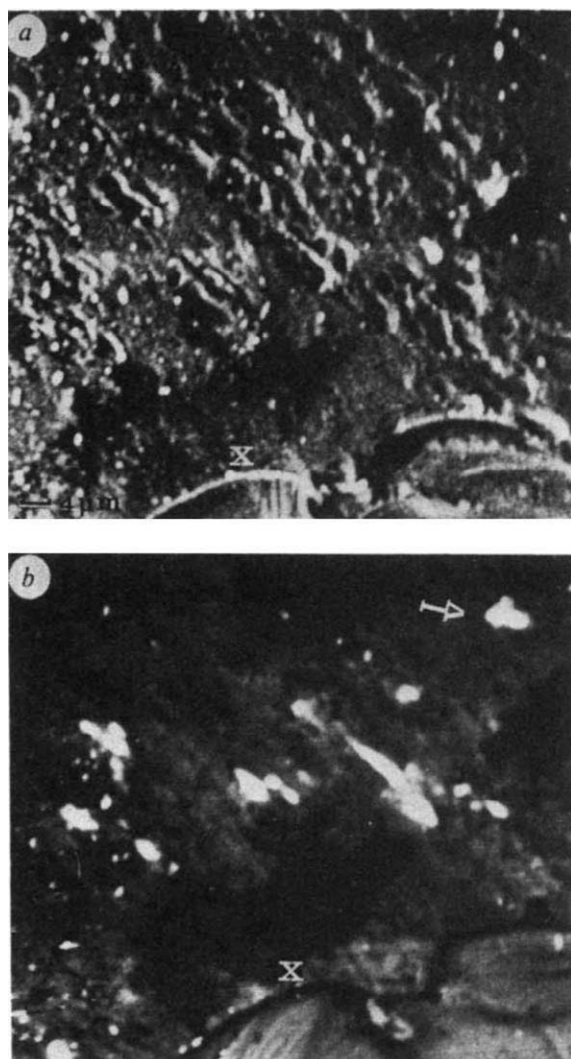


Fig. 1 Reduced TiO_2 : a, SEM image; b, cathodoluminescence image. Liquid N_2 temperatures were used.

experiments were also performed outside the HVEM in a gas reaction chamber connected to a mass spectrometer and a gas chromatograph, to confirm the direct studies. Cathodoluminescence experiments were carried out on both fresh and reacted specimens using a Cambridge Stereoscan SEM fitted with a cathodoluminescence detector and operating at 30 keV. A combination of an elliptical mirror and light guide was used to collect the cathodoluminescence with reasonable efficiency. It could be connected either directly to a suitable photomultiplier for the total signal or through an optical spectrometer, which was scanned to produce the analytical spectra¹².

In general, the prepared materials produced relatively uniform images in both SEM (secondary electron image and back-scattered electrons and cathodoluminescence), which were interpreted as coming from smooth and homogeneous surfaces. After reaction, considerable surface roughness was often imaged in the SEM as shown in Fig. 1a for rutile reduced at 850 °C; notable and unexpected cathodoluminescence contrast effects were obtained (Fig. 1b), which cannot be readily explained by the observed structural defects or surface topography, that is, they are not well correlated with the SEM images from the same areas. Figure 1b illustrates sharply defined patches of bright contrast and also the darker inverse (with respect to the background level).

The energy of the cathodoluminescence signal is a function of the bandgap for the appropriate transition between electron energy states in the material. Using this technique, it has been possible to confirm already known bandgaps⁹, for example,

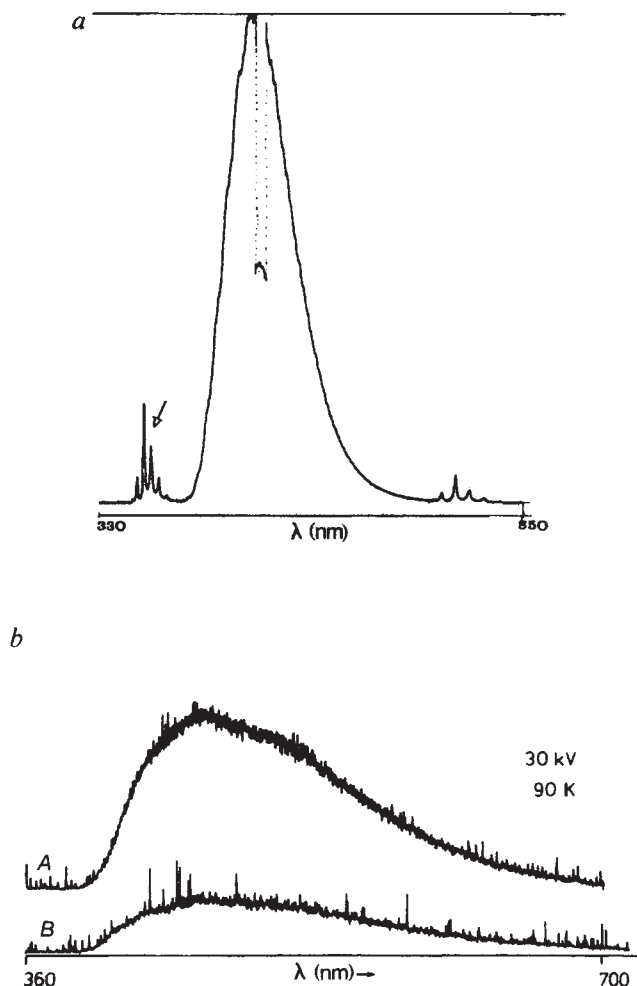


Fig. 2 a, Cathodoluminescence spectrum of pure ZnO, showing the potential of the technique. The bandgap is 3 eV (arrowed) but donor levels (of 0.3 eV) below the conduction band create additional charge carriers. b, Spectra from two regions of reduced TiO_2 each $10 \mu\text{m}^2$. A is from a bright region (as arrowed in Fig. 1) and B is from a background region. The difference is mainly owing to point defects. The peak occurs at 465 nm (2.67 eV).

~3 eV for ZnO (Fig. 2a) and 3.2–3.5 eV for TiO_2 used as a support for catalysts, and to establish completely unknown electron energy levels from the emission colour and spectra of small reacted crystallites of TiO_2 , even from the selected ~10- μm^2 regions shown in Fig. 2b. Cathodoluminescence experiments have also been useful in the interpretation of catalytic behaviour of complex mixed Sn/Sb oxide (with 0.7 atom % Sb) catalysts.

In situ H_2 -reduction experiments carried out on the catalyst system in the HVEM show no extended CS defects at operating temperatures. The SEM images of the reacted specimen shown in Fig. 3 indicate considerable complexity, with in this case evidence for segregation of species on the surface. The cathodoluminescence spectra of the reacted and unreacted specimens reveal marked differences as shown in Fig. 4A and B respectively, with band gaps of 2.6 eV and 1.9 eV, suggesting variations in composition. The EPMA quantitative analysis shows depletion of Sb in the bulk (0.2 atom % Sb in the bulk) and, therefore, possible surface segregation of Sb. This work has also produced the first estimates of bandgap energy of ~2 eV for Bi_2MoO_6 (γ -bismuth molybdate), which is the commercially important catalyst in the selective oxidation/ammoxidation of propylene to acrolein/acrylonitrile used for resins and fibres¹. This energy (2 eV) is conveniently in the visible region of the spectrum.

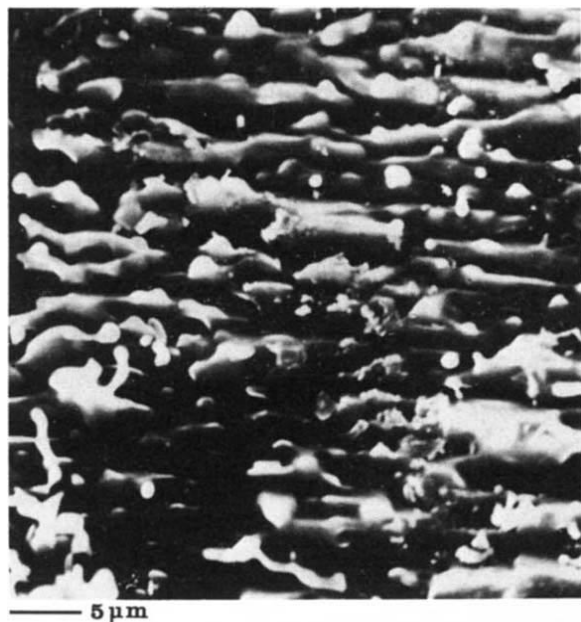


Fig. 3 SEM image of Sn/Sb oxide catalyst reduced in H_2 , indicating surface segregation of species.

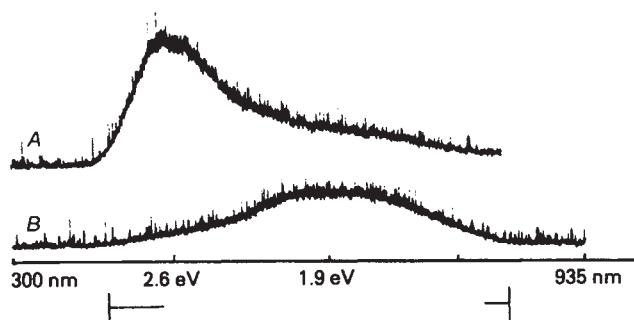


Fig. 4 Cathodoluminescence spectra of A, the reacted Sn/Sb oxide catalyst and B, the unreacted catalyst, showing a peak shift indicative of chemical or structural change.

The energy and intensity of the cathodoluminescence signal is influenced by structural features that modify electronic properties including the generation of defects and new phases under operating conditions. The allowed electron energy levels can be expected to be changed by the presence of the defects. If discrete levels, perhaps associated with structural changes, are involved, a different and diagnostic wavelength of radiation may be produced. Point defects can introduce a continuous range of degenerate levels leading to broadening of the emission peaks (for example, Fig. 2b) and to a progressive and non-radiative quenching of the diffusing carrier species before a radiative recombination process can occur. Alternatively, point defects may act as carrier traps, enhancing the probability of radiative recombination in a way similar to activators in phosphors and dopants in light emitting diodes. This might also be one role of the promoters present in many commercial catalysts and could help to explain the mechanism by which they are effective.

The image contrast obtained with cathodoluminescence can thus be used to examine the distribution of moderate to low levels of point defects in the matrix, and in the presence of more extended defects, which are believed to play a major role in both binding of reaction species and in diffusion through the bulk between the reaction sites. To minimize the introduction of additional point defects, cathodoluminescence microscopy was always performed before other examinations at higher voltages. More efficient cathodoluminescence detection should reduce the beam current requirements and the risk of any artefact

effects being induced under vacuum in the SEM. All the cathodoluminescence results have been obtained with normal incidence at 30 keV. Lower voltages and operation with glancing incidence in a manner analogous to reflection high-energy electron diffraction (RHEED) should permit more surface localized analysis, although there will also be enhanced recombination at the surface, which will reduce intensities.

The results should assist in the explanation and prediction of the catalytic properties of some complex and not well understood but commercially important materials.

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Received 8 May; accepted 19 December 1984.

1. Hucknall, D. J. in *Selective Oxidation of Hydrocarbons* (Academic, New York, 1974).
2. Stone, F. S. *J. Solid State Chem.* **12**, 271-281 (1975).
3. Gai, P. L., Boyes, E. D. & Bart, J. C. *J. Phil. Mag.* **A45**, 531-547 (1982).
4. Gai, P. L. *J. Solid State Chem.* **49**, 25-42 (1983).
5. Gai, P. L. *Phil. Mag.* **A43**, 841-857 (1981).
6. Holt, D. B. *Inst. Phys. (Lond.) Conf. Ser.* **60**, 165-178 (1981).
7. Booker, G. R. *Inst. Phys. (Lond.) Conf. Ser.* **60**, 203-214 (1981).
8. Boyes, E. D. & Gai, P. L. *Proceedings of AEM workshop, Microbeam Analytical Society, Vail* (ed. Geiss, R.) 71-75 (San Francisco Press, San Francisco, 1981).
9. Rao, C. N. R. & Subbarao, G. V. *Phys. Stat. Sol. A1*, 597-614 (1970).
10. Blanchin, M., Bursill, L. A., Hutchison, J. & Gai, P. L. *J. Physique* **42**, 95-110 (1981).
11. Mackrodt, W. C. in *Computer Simulation in the Physics and Chemistry of Solids*, 71-82 (Daresbury Laboratory, 1980).
12. Warwick, C. A. & Booker, G. R. *Inst. Phys. Conf. Ser.* **67**, 321-326 (1983).

Surface retexturing of Pt wires during the catalytic oxidation of CO

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The catalytic properties of Pt, which have been studied in a variety of forms of the metal (single crystals, wires or dispersed on a support), may be influenced by the method of preparing the metal surface. Pt surfaces undergo retexturing¹ during catalytic reactions at high temperatures (>1,000 K) and a relatively facile sintering process has also been described². Engel and Ertl³ have reviewed the heterogeneous oxidation of carbon monoxide on Pt ($CO + \frac{1}{2}O_2 \rightarrow CO_2$), emphasizing studies concerned with the behaviour of the reaction at very low pressures and on clean surfaces (for example, on single crystal faces). Under these conditions the kinetic behaviour is determined by the ratio of the partial pressures of the reactants and is relatively insensitive to the total pressure. Here, we describe microscopic and kinetic observations of the oxidation on Pt wire surfaces below 600 K and at atmospheric pressure. The surface reactions are apparently more complicated here than at low pressure; extrapolations from the low to the high pressure regimes may not be applicable because of changes in the rate-controlling parameters. We present evidence of the mobility of Pt atoms (often considered immobile) on the surface and suggest that the catalyst functions through formation of oxy- and/or carboxyl-Pt complexes in a two-dimensional chemisorbed layer, envisaged as a monolayer possessing fluid properties and enhanced reactivity, containing both adsorbed reactant gases and mobile catalyst atoms.

Our catalysts are in the form of multicrystalline wires (11-turn helical coil wound from 50-μm diameter wire; Johnson Matthey, Thermopure Grade) which are particularly suitable for characterizing the surface textural changes accompanying their participation in CO oxidation. Surfaces were examined directly in a scanning electron microscope (Jeol 35CF) after the wires had been used in rate studies involving a selected sequence of kinetic measurements under controlled conditions (wire temperature, reactant compositions and duration of reaction).

Kinetic measurements of the CO oxidation reaction were made by a novel method⁴⁻⁶ outlined here. The catalyst wire coil, also used as its own resistance thermometer, was first calibrated at pre-determined temperatures, usually 450-550 (±0.5) K, in a

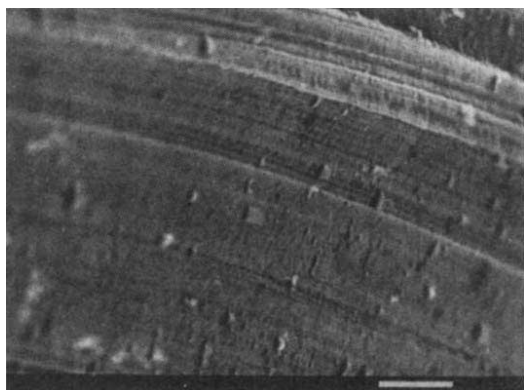


Fig. 1 Typical texture of pristine Pt wire before use in heterogeneous catalytic reaction. Scale bar, 1.0 μm .

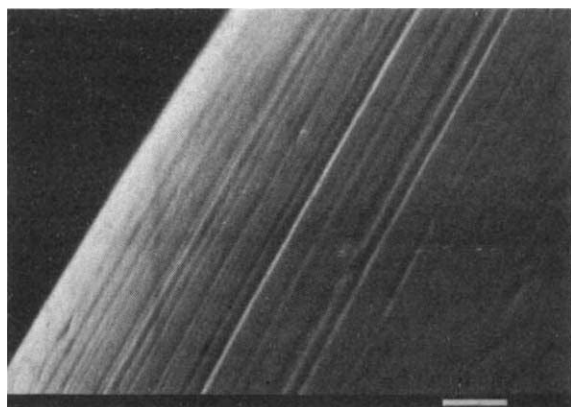


Fig. 2 Texture of Pt wire after heating to $\sim 1,200$ K for 850 min in a flow of O_2 -55 and N_2 -250 $\text{cm}^3 \text{min}^{-1}$. Surfaces are smoother than those of the pristine wire (Fig. 1), but surface striations are still easily visible. Scale bar, 1.0 μm .

carefully controlled constant rate of flow of non-reactive gas mixture (O_2 , 55; N_2 , 245 $\text{cm}^3 \text{min}^{-1}$, ambient temperature and atmospheric pressure). The power, P_C , supplied to maintain a constant total resistance, and hence a constant mean wire temperature, was controlled electronically at any desired value through a resistance bridge and feedback circuit. Effects tending to induce a change in wire resistance (and, thereby, temperature) were compensated automatically by a change in the power supplied. When a constant flow of a reactive gas mixture (O_2 , 55; CO , 5) 10 or 20 $\text{cm}^3 \text{min}^{-1}$ and sufficient N_2 diluent to maintain the original balance) replaced the inert ($\text{O}_2 + \text{N}_2$) mixture, a lower power, P_R , was required to maintain the wire temperature at the same value. The difference in power supplied, $\Delta P = P_C - P_R$, is a measure of the heat release rate by reaction ($\text{CO} + \frac{1}{2}\text{O}_2 \rightarrow \text{CO}_2$) at the constant temperature of the catalyst. Verifications of the wire calibration P_C were repeated frequently during the kinetic studies at appropriate temperatures. The merit of these gas mixtures ($\text{O}_2 + \text{N}_2$ and $\text{O}_2 + \text{CO} + \text{N}_2$) is that neither changes in the reactant proportions, nor changes to the non-reactive calibrant, have significant effect on the heat-transfer properties of the flowing gas.

Results of the relevant kinetic studies are summarized. There are two distinct regimes of reaction under oxygen-rich conditions, one accessible only by raising the wire temperature from below ~ 400 K and characterized by a relatively low rate of heat release from the surface ($\Delta P < 50$ mW). We shall term this the kinetic regime, as it is believed that this reaction is controlled by surface rate processes involving only chemisorbed species. Kinetic studies were made between 460 and 520 K and the activation energy was determined (140–210 kJ mol^{-1}). The temperature over which kinetic control operates is limited, as there

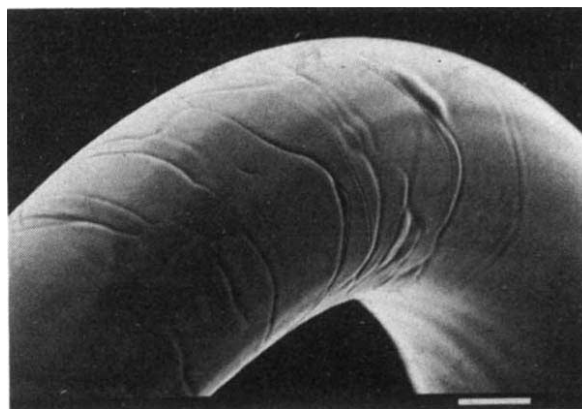


Fig. 3 After stronger heating (perhaps 1,800 K for 60 min) (compare Fig. 2) the wire surface was almost smooth, although now textured by a net of shallow depressions identified as grain boundaries. Scale bar, 10 μm .

is a discontinuous transition to a regime of markedly enhanced reactivity ($\Delta P \approx 150$ mW) as the wire temperature is raised to ~ 550 K. This second reaction regime is believed to be under diffusion control⁴; the wire temperature may be raised or lowered without markedly changing the rate of heat release. If the temperature is diminished to ~ 500 K, however, there is another sharp discontinuity (extinction) which returns reaction to the kinetic regime. The diffusion regime can be entered thereafter only by raising the temperature again until the upward transition in power output is effected once more. The kinetics of reaction thus exhibit hysteresis and there is a region of bistability within which the reaction rate may be controlled by either the kinetic or the diffusion limiting processes.

Representative textural features of pristine wires are illustrated in Fig. 1. The parallel surface striations were introduced, presumably, during the wire drawing. All surfaces were irregular with numerous small and often rounded protrusions ($< 0.1 \mu\text{m}$), the sizes, numbers and dispositions of which varied locally. The appearances of different areas were broadly similar.

Wires heated for some hours at 1,200 K in a flow of a non-reactive gas mixture (O_2 , 55 and N_2 , 250 $\text{cm}^3 \text{min}^{-1}$) were perceptibly less rough than the pristine wires, though the striation structure persisted (Fig. 2). After still stronger heating, as judged from the perceived radiation, the surfaces of the hottest central portion of the coil had become very smooth (Fig. 3) and were patterned with a network of shallow depressions identified as grain boundaries. The outer portions of such coils, cooled by heat losses through the heavier support wires, underwent less change.

Wires used for the heterogeneous catalytic oxidation of CO for some hours at ~ 500 K developed the characteristic surface textures shown in Fig. 4 a-c. Identical structural features were observed on each of four different wires during detailed and systematic examinations in the scanning electron microscope. These examples of surface features were selected from 210 photomicrographs as being representative and characteristic. The surface textural features (Fig. 4) developed only when the Pt wire had been exposed to O_2 and CO simultaneously and after their partial conversion to CO_2 had continued for some hours at ~ 500 K. The wires used here were never heated above 600 K. No indication of surface retexturing could be discerned (at magnifications of up to 30,000) in the absence of the catalytic reaction.

The surface restructuring described here bears striking similarities to the retexturing reported⁷ for the oxidation of ammonia on Pt. The arrays and dispositions of facets, in Fig. 4, resemble the patterns illustrated in Fig. 2a, b of ref. 7. The dimensions of facets developed during the CO oxidation at ~ 500 K (0.1–0.5 μm) were significantly smaller than those

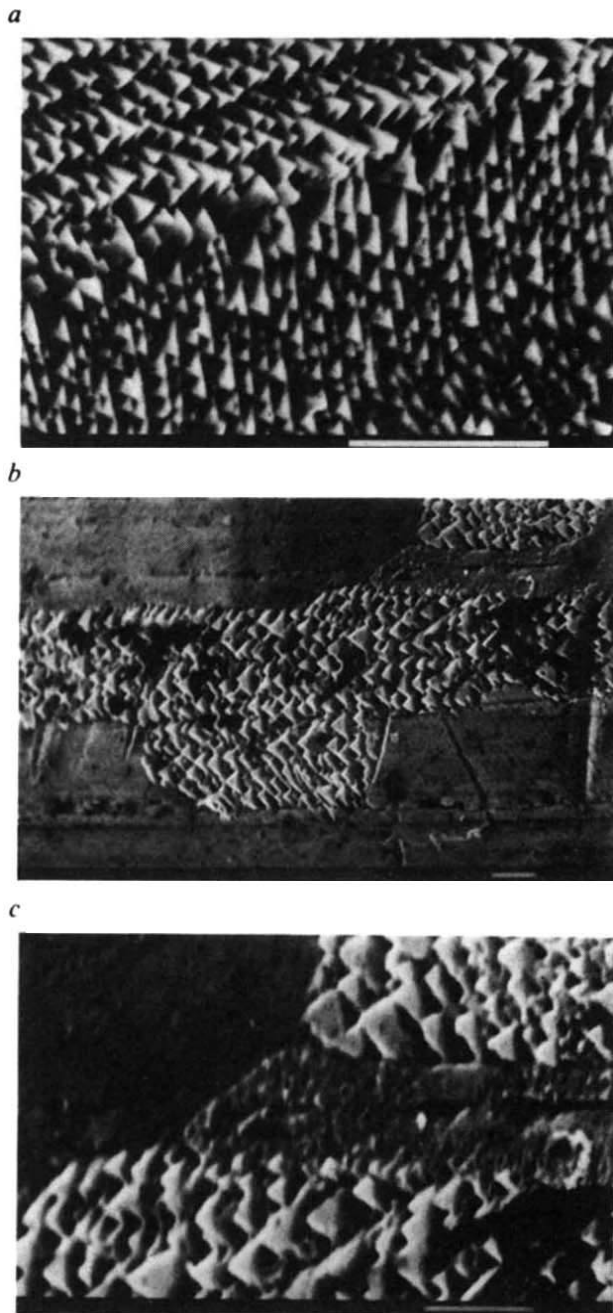


Fig. 4 Characteristic textures developed on Pt wire surfaces after several hours of catalytic oxidation of CO, below 600 K. Surface reorganization involved the generation of crystallographic facets, identified as (100) and/or (111) faces together with some rounding of corners. Facet development on adjoining areas was sometimes oriented in different directions, which could be because of the multicrystalline structure of the wire. Retexturing was usually a localized phenomenon; textured zones often adjoining areas that had undergone little or no apparent change. *a*, Pt wire surface after 30-h reaction ($\text{CO} + \frac{1}{2}\text{O}_2 \rightarrow \text{CO}_2$) at ~ 500 K. *b*, *c*, Surface after 430-h reaction at 480–560 K (*c* is a detail of *b*). Scale bars are all 1.0 μm .

observed in the higher temperature ($> 1,000$ K) ammonia oxidation (0.5–3 μm).

From X-ray energy dispersive and wave dispersive analyses of four retextured wires it has been established unequivocally that in the outer layer of the wire (~ 1 μm) there is on average 6–8% oxygen, which corresponds to a surface layer composition approximating to PtO. The same compositions (PtO) were detected at both textured and unchanged areas of wires after use as catalysts and also at the surface of pristine wires.

We propose the following explanation to account for these observations. During the heterogeneous oxidation of CO, Pt surfaces undergo locally variable retexturing; some areas experience complete modification through the development of crystal facets and other areas are manifestly unchanged. It seems probable that recrystallization occurs only at the surfaces of those Pt crystals of the wire that are suitably oriented and that the textural discontinuities (Fig. 4) are due to grain boundaries. Facet faces are identified, from symmetry considerations, as high occupancy surfaces of the Pt metal structure (face-centred cubic), probably (111) and/or (100).

These observations lead us to two important considerations in the formulation of a reaction mechanism for CO oxidation. The first is the demonstration that the Pt exhibits significant surface mobility at temperatures (~ 500 K) very far below the melting point of the metal (2,045 K). The second is that a bulk platinum oxide (rather than adsorbed oxygen) may be the solid actively promoting, or exerting a controlling influence on, the present reaction.

These considerations are consistent with two alternative mechanistic representations of the present reaction. In one, surface⁸ or multilayer oxide is reduced by chemisorbed CO and replenished from dissociatively adsorbed oxygen. CO_2 formation occurs at the contact interface between metal covered with CO and the metal oxide⁹, a mechanism strongly resembling certain solid state decomposition reactions¹⁰. In the other representation, the metal atoms are regarded as entering a mobile chemisorbed phase which resembles a two-dimensional fluid of molecular thickness. In this phase, Pt bonds with O_2 and/or CO (with transitory carboxyl, oxy- or carbonyl-compound formation) to produce labile intermediates that are formally analogous to those envisaged as participants in homogeneous reactions in a solvent. The important difference here is that the 'homogeneous' medium is two dimensional, of molecular thickness and its components remain bonded to the supporting active surface.

The precise role of any oxide in these reactions must remain conjectural until the chemistry of the various platinum-oxygen (-carbon) species can be established satisfactorily. The chemical behaviour described for the compounds presently known evidently excluded their possible participation in this catalytic reaction. The reduction of PtO_2 with CO is particularly vigorous; we have measured a temperature rise to > 430 K soon after exposing a 0.1-g compact sample of PtO_2 to a flow of CO at ambient temperature, which confirms the much earlier observations by McKinney¹¹. Other oxides of Pt, stable at higher temperatures, have been described^{12,13}. There is evidence from rate studies^{3,14} and from oxygen isotopic exchange measurements¹⁵ that the reactivity of oxygen and/or oxide at Pt surfaces increases at ~ 500 K, the temperature of the present reaction. The chemistry of the platinum oxides, however, requires further investigation.

The observed surface retexturing is evidence of the complexity of the surface interactions, which also result in hysteresis in kinetic behaviour and oscillatory reactions⁶. We must conclude that the surface concentrations of active participants are sensitive to prevailing conditions and previous catalyst treatment. It is perhaps significant that relatively few values of the activation energy for CO oxidation on Pt are to be found in the literature. Analysis of our kinetic data yielded pre-exponential factors (10^{36} – 10^{43} molecules $\text{m}^{-2} \text{s}^{-1}$) appreciably greater than the value expected for a surface reaction (10^{32} molecules $\text{m}^{-2} \text{s}^{-1}$), which may be ascribed to temperature dependent changes in the concentrations of surface participants¹⁶.

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1. Flytzani-Stephanopoulos, M., Wong, S. & Schmidt, L. D. *J. Catal.* **49**, 51–82 (1977).
2. Baird, T., Paál, Z. & Thomson, S. J. *J. chem. Soc., Faraday Trans. 1* **69**, 50–55 (1973).
3. Engel, T. & Ertl, G. *Adv. Catal.* **28**, 1–78 (1979).

4. Gray, P., Griffiths, J. F. & Rogerson, J. S. *Am. Soc. Mech. Engng* 79-HT-56, 1-8 (1979).
5. Jones, A., Firth, J. G. & Jones, T. A. *J. Sci. Instrum.* 8, 37-40 (1975).
6. Rogerson, J. thesis, Univ. Leeds (1983).
7. McCabe, R. W., Pignet, T. & Schmidt, L. D. *J. Catal.* 32, 114-126 (1974).
8. Barteau, M. A., Ko, E. I. & Modix, R. J. *Surf. Sci.* 104, 161-180 (1981).
9. Gland, J. L. & Kollin, E. B. *J. chem. Phys.* 78, 963-974 (1983).
10. Galwey, A. K. *Thermal Analysis, Proc. 7th Int. Conf.*, 38-53 (Wiley, Chichester, 1982).
11. McKinney, P. V. *J. Am. chem. Soc.* 56, 2577-2580 (1934).
12. Ducros, R. & Merrill, R. P. *Surf. Sci.* 55, 227-245 (1976).
13. Berry, R. J. *Surf. Sci.* 76, 415-442 (1978).
14. Bonzel, H. P. & Ku, R. J. *Vac. Sci. Technol.* 9, 663-667 (1972).
15. Borekov, G. K. *Adv. Catal.* 15, 285-339 (1964).
16. Galwey, A. K. *Adv. Catal.* 26, 247-322 (1977).

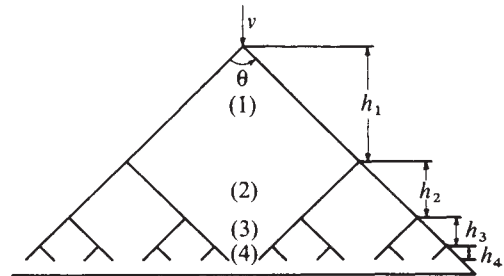


Fig. 1 Illustration of a fractal tree. At each level n there are 2^n legs. The height of the n th level is $h_n = h_1/2^{n-1}$. In the limit $n \rightarrow \infty$, the total height $h \rightarrow 2h_1$. The tree is loaded with the vertical force V .

Collapse of loaded fractal trees

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Mandelbrot¹ has argued that a wide range of natural objects and phenomena are fractals; examples of fractal trees include actual trees, plants such as a cauliflower, river systems and the cardiovascular system. Here we apply the renormalization group approach² to the collapse of fractal trees, which may be applicable to a variety of problems including cardiac arrest, failure of bronchial systems, failure of electrical distribution systems and the instability resulting in earthquakes.

As a simple example of a fractal tree we consider the two-dimensional structure shown in Fig. 1 and its failure under the application of a vertical load V . Without any failure, the load on each leg of order n is $V_n = V/2^n \cos \theta/2$. We hypothesize that the legs have a statistical distribution of strength; the probability ${}^n P_1$ that the failure strength of a n th level leg, V_{nt} , is less than or equal to the load V_n is

$$\text{Prob}(V_{nt} \leq V_n) = {}^n P_1 = 1 - \exp[-(V_n/V_{n0})^m] \quad (1)$$

where V_{n0} is a reference strength of the n th level legs and m is an integer. This is known as a Weibull distribution and is often used to represent a statistical distribution of failure strengths³.

We further hypothesize that if one leg fails, the load applied to that leg is transferred to the adjacent leg in the pair; the second leg may suffer an induced failure which we describe by the conditional probability ${}^n P_{2,1}$ that failure will occur when a stress V_n is transferred to an unbroken leg, already supporting a stress V_n , given by⁴

$$\begin{aligned} {}^n P_{2,1} &\equiv \text{Prob}(V_{nt} \leq 2V_n/V_{nt} > V_n) \\ &= \frac{\text{Prob}(V_n < V_{nt} \leq 2V_n)}{\text{Prob}(V_{nt} > V_n)} \\ &= \frac{{}^n P_2 - {}^n P_1}{1 - {}^n P_1} \end{aligned} \quad (2)$$

where ${}^n P_2 = 1 - \exp[-(2V_n/V_{n0})^m]$ which becomes, using equation (1), ${}^n P_2 = 1 - (1 - {}^n P_1)^{2^m}$.

We now consider the failure of a pair of legs at the n th level, which, because it removes the support of the $(n-1)$ -level leg immediately above, is equivalent to the failure of a single leg at the $n-1$ level and is thus denoted by ${}^{n-1} P_1$. The direct failure of a pair of legs is given by ${}^n P_1^2$ and by including induced failures we obtain

$$\begin{aligned} {}^{n-1} P_1 &= {}^n P_1^2 + 2^n P_1(1 - {}^n P_1){}^n P_{2,1} \\ &= 2^n P_1 {}^n P_2 - {}^n P_1^2 \\ &= 2^n P_1[1 - (1 - {}^n P_1)^{2^m}] - {}^n P_1^2 \end{aligned} \quad (3)$$

It is implicit in the renormalization group approach that our failure condition, equation (3), is applicable at all levels of the structure. The hypothesis of scale invariance, the basis of this

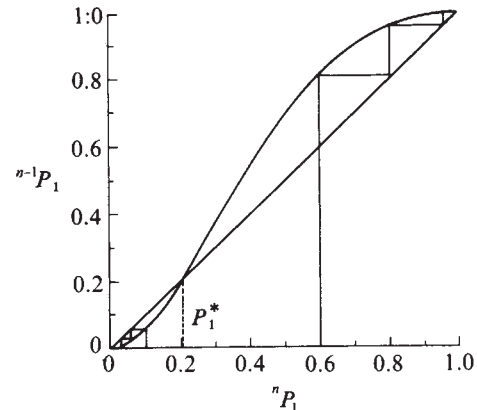


Fig. 2 Dependence of the probability for the failure of a leg at the $n-1$ level, ${}^{n-1} P_1$, on the probability of failure at the n th level ${}^n P_1$ from equation (3). The procedure described in the text for determining failure at successive levels is illustrated for ${}^n P_1 = 0.6$ and 0.1. The points 0 and 1 are stable fixed points of the system. The straight line corresponds to ${}^{n-1} P_1 = {}^n P_1$, and the failure condition crosses this at $P_1^* = 0.2063$. The critical probability of failure P_1^* gives the bifurcation point for catastrophic failure of the system.

approach, implies the existence of a unique model of the system that is independent of the length scale except for a prescribed change in the pertinent parameters. In this case the scale invariance is implemented through a statistical distribution of asperity strengths that is invariant, in that it is the same for asperities of all orders, except for an overall renormalization of stress.

As a specific example we take $m = 2$ for which the dependence of ${}^{n-1} P_1$ is given in Fig. 2. A renormalization group iteration can be performed graphically using Fig. 2. For example, we take ${}^n P_1 = 0.6$ and from equation (3), with $m = 2$, we find ${}^{n-1} P_1 = 0.8093$; applying equation (3) iteratively, we find ${}^{n-2} P_1 = 0.9615$, which can be obtained graphically in Fig. 2 by extending a horizontal line to the line ${}^{n-1} P_1 = {}^n P_1$ and then a vertical line to the S-curve representing the solution to equation (3). The iteration is then repeated to give ${}^{n-3} P_1 = 0.9985$; thus the probability of failure rapidly approaches unity as n is decreased. If we take ${}^n P_1 = 0.1$, however, we find that ${}^{n-1} P_1 = 0.05878$, ${}^{n-2} P_1 = 0.02181$, ${}^{n-3} P_1 = 0.00322$, etc. and the probability of failure approaches zero as n is decreased. Thus, if ${}^n P_1 < P_1^*$ the structure is stable, but if ${}^n P_1 > P_1^*$ the structure will collapse—a catastrophic failure will occur.

From equation (1), for $n = 2$, we find that the failure load V^* corresponding to $P_1^* = 0.2063$ is $V^* = 0.4807 V_{n0}$, which is

Table 1 Critical probability of failure and failure load

m	P_1^*	V^*/V_{n0}
2	0.206	0.481
3	0.094	0.462
5	0.0221	0.467
10	0.00068	0.482

considerably less than the mean strength of a n th level leg $\bar{V} = 0.8862 V_{n0}$. These results are summarized in Table 1 together with those for $m = 3, 5$ and 10 . As m increases, the distribution of failure loads is reduced and in the limit $m \rightarrow \infty$ all legs will fail when $V_n = V_{n0}$; as m increases, the critical probability for failure rapidly decreases, but the failure load V^* is almost independent of m .

Although we have considered the failure of a particular fractal tree, we believe that our approach is appropriate for the failure of a network of systems in which the probability of failure is independent of the level in the network. Consider the n th level of the fractal tree illustrated in Fig. 1. A leg at this level represents a system that includes the legs at all the lower levels and as long as the systems at all levels obey the same probability law, for example that given in equation (1), then the renormalization group approach can be applied. With the transfer of load to the neighbouring system the characteristic S curve of catastrophic failure is observed.

In conclusion, we have obtained the following general results,

which may be applicable to a variety of problems: (1) the total system will fail at a lower load than the sum of the mean failure loads of the sub-systems; (2) reducing the deviation in failure loads for sub-systems will not increase the failure load of the entire system. This approach to the stability of a fractal tree was derived to explain the earthquake instability⁵ (the legs of the fractal tree are the asperities and barriers on a fault in this example) but other applications may include cardiac arrest and the failure of bronchial systems, that is, the collapse of actual fibrous materials, because the fibres generally have equal diameters, our results may suggest improved fibrous materials with a statistical distribution of diameters. Our results may also be applicable to the reliability and safety of control systems and electrical distribution systems.

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Received 25 September; accepted 23 November 1984.

1. Mandelbrot, B. *The Fractal Geometry of Nature* (Freeman, San Francisco, 1982).
2. Wilson, K. G. & Kogut, J. *Phys. Rep. C* **12**, 75–200 (1974).

3. Harlow, D. G. & Phoenix, S. L. *Adv. Appl. Probability* **14**, 68–94 (1982).
4. Meyer, P. L. *Introductory Probability and Statistical Applications* (Addison-Wesley, Reading, 1970).
5. Smalley, R. F., Turcotte, D. L. & Solla, S. A. *J. geophys. Res.* (in the press).

Origin of agates in volcanic rocks from Scotland

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Silica, SiO_2 , is found in nature in a wide range of forms from amorphous to crystalline varieties. Minerals composed mainly of microcrystalline quartz (MQ) are conveniently characterized by the chemistry or mineralogy of accessory constituents and by crystallographic features of the quartz^{1–5}. Agates are MQ nodules of high purity found in cavities in host rocks of volcanic origin. A recent theory¹ described agate growth as a sequence of events whereby initial chalcedony (fibrous length-fast MQ) is deposited from a supercritical fluid that is poorly polymerized; later, spherulitic quartzine and chalcedony form from a highly polymerized siliceous liquid or gel via its transformation into horizontally layered opal-CT and fine-quartz. As cavity filling is considered a consequence of pressure release on cooling, it is implied that temperatures were reasonably high. We present here isotope ratios, $^{18}\text{O}/^{16}\text{O}$ of silica and D/H of bound water, in agates from Lower Devonian (~410 Myr BP) lavas; these ratios are distinct from those in Tertiary (~62 Myr BP) lavas, but are linearly correlated and plot along a single line approximately parallel to the line defined by present-day meteoric waters. The data imply that bound waters associated with the agates have preserved their hydrogen isotope ratios since agate deposition, which supports arguments that the water content is of genetic significance¹, but does not support a model for agates as 'refused' chert xenoliths². We infer that agates of both ages were formed at low temperature (~50 °C) from fluids having at least a component of meteoric origin.

Agate formation temperatures >375 °C were proposed⁶ on the basis of a crystallite size geothermometer. Data on agate geochemistry⁷ and stable oxygen isotope ratios² have also been reported. Preliminary $\delta^{18}\text{O}$ values (in ‰ relative to SMOW—Standard Mean Ocean Water) on 13 agates ranged from 22.4 to 31.3‰ (ref. 2) and it was argued that such values were unlikely to result from post-crystallization processes. Because of the similarity of this range with that for marine cherts, it was

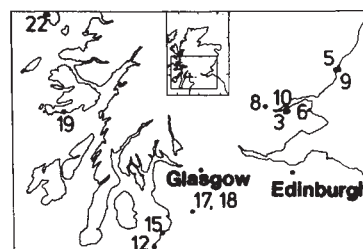


Fig. 1 Location of Scottish agates described in Table 1. The agates from the Lower Devonian were collected over a wide area. The localities are approximate as most of the samples were found loose. The specimens from the Tertiary lavas in Mull were all taken from the rock at a single locality, which falls outside the area of hydrothermal alteration in the centre of the island, but where sills in the basalt are made of pitchstone or contain sedimentary xenoliths.

postulated² that agates in volcanics are xenoliths of marine chert that are not resorbed by the magma, but are carried within it as melt drops and thus transformed to agate (see also ref. 8).

Knauth and Epstein⁹ have argued that nodular and bedded cherts (identified as granular microcrystalline quartz) contain water as hydroxyl groups (—OH) and that the hydrogen isotopic composition (δD) of this 'water', like the oxygen isotope ratio of the silicate, is preserved with time. Similar cherts from differing geological epochs were claimed to fall into broad distributions approximately parallel to the present-day meteoric water line (MWL), defined by $\delta\text{D} = 8\delta^{18}\text{O} + 10$. Variations in the position of the chert line (that is, the intercept of the δD against $\delta^{18}\text{O}$ plot) were interpreted as reflecting earth-surface temperature changes. Related work on deep-sea cherts^{10,11} confirmed the retention of an original δD signature in various phases, including chalcedony formed on the ocean floor.

We have measured $\delta^{18}\text{O}$ of silicate oxygen and δD of water (released by pyrolysis above 120 °C and denoted H_2O^+) on selected agates from Lower Devonian lavas from Scotland. Specimens showing both wall banding and horizontal banding were included. Sample descriptions and data are given in Table 1 and the locations of the agates found are shown in Fig. 1. For 13 samples there is a variation in $\delta^{18}\text{O}$ of +20–26‰, water contents (H_2O^+) range over ~0.1–0.5 wt % and δD , –60–110‰. There is no relationship between δD and H_2O^+ . Figure 2, which plots δD against $\delta^{18}\text{O}$, shows that except for the two samples JJ12 and JJ17 there is a strong positive correlation. The

Table 1 Isotope ratios for agates in volcanic rocks from Scotland

Sample	Location, collector and grid ref.	$\delta^{18}\text{O}$ (‰ V-SMOW)	δD (‰ V-SMOW)	H_2O^+ (wt %)
JJ1 (H)	Devonian; exact location not known, probably Ayrshire	24.9; 24.6	-60	0.26
JJ2	Devonian; Fife near samples 3, 6 and 10	20.5	-94	0.20
JJ3	Devonian; Dunbog; C.C.; NO 2818	21.8	-97	0.17
JJ4	Devonian; exact location not known, probably Fife	20.2	-103	0.53
JJ5 (H)	Devonian; Usan; C.C.; NO 7254	23.1	-73; -66	0.43; 0.44
JJ6	Devonian; Balmeadowside; C.C.; NO 3118	21.4	-93; -91	0.11; 0.12
JJ8	Devonian; Kinnoull Hill; C.C.; NO 1322	21.7	-100	0.13
JJ9 (H)	Devonian; Boddin Point; C.C.; NO 7153	23.7	-80; -85	0.39; 0.42
JJ10	Devonian; Pittachope; C.C.; NO 3021	20.9	-99	0.33
JJ15 (H)	Devonian; Dunure; J.J.; NS 2515	21.7	-73; -71	0.42; 0.44
JJ18	Devonian; Burn Anne; GS; NS 5235	22.1	-78	0.41
JJ12	Devonian; Turnberry; J.J.; NS 2005	25.8; 25.6	-107; -107	0.15; 0.18
JJ17	Devonian; as JJ18	24.4; 23.4	-111; -117	0.17; 0.15
JJ19	Tertiary; Mull; B.J. and W.J.B.; NM 4519	13.6	-153	0.47
JJ20A	Tertiary; Mull; as JJ19	13.2	-174	0.15
JJ20B	Tertiary; Mull; as JJ19	16.8	-136	0.68
JJ22	Tertiary; Rhum; G.D.; NG 3201	13.4	-140	0.64
JJ24	Tertiary; Mull; as JJ19	15.6	-134	0.45
JJ25	Tertiary; Mull; as JJ19	15.2	-129	0.45
JJ26	Tertiary; Mull; as JJ19	15.6	-135	0.41

Horizontally banded agates were distinguished from the normal fortification variety and are denoted by the letter H in column 1. Under the microscope the chalcedony was length-fast; the horizontal layers were either fibrous length-fast chalcedony or a granular material, which was not treated separately. Opaline silica was sought and was found in one of the Tertiary samples. This specimen JJ20 had a distinct outer layer (JJ20B) which was separated and identified as cristobalite by X-ray diffraction. With this exception the material all consisted of chalcedony with slight iron-staining as the only sign of impurity. The collectors are identified by their initials (see acknowledgements).

two discrepant agates have the highest $\delta^{18}\text{O}$ and the lowest δD of all the Devonian samples analysed; there are yet no valid criteria to distinguish them on other than isotopic grounds. Linear regression of the 11 remaining points gives $\delta\text{D} = 8.4\delta^{18}\text{O} - 271$; the correlation coefficient $r = 0.82$ is significant at the 99% level. The first conclusion is that H_2O^+ is related to the last fluids with which the agate silicates exchanged oxygen isotopes. To investigate the possibility that the interaction with fluids took place long after deposition (and thus had no bearing on agate genesis), Tertiary agates from the Western Isles of Scotland were examined. Host volcanics are aged ~62 Myr BP and contemporary meteoric fluids are known to have been strongly depleted isotopically, probably because of a combination of geographical and topographical features¹²⁻¹⁴; the position of such fluids on the MWL has been estimated as (-12%, -85%) (see, for example, ref. 14). The isotope ratios for Tertiary agates are much lighter than those from the Lower Devonian. We interpret this as evidence that interaction with recent waters has not been significant and that the isotopic signatures of the agates were established during or soon after formation. The Tertiary points fall close to the extrapolation of the best-fit line through the Devonian data (see Fig. 2). Pooling the 18 points leads to a regression line $\delta\text{D} = 7.9 \delta^{18}\text{O} - 260$, with $r = 0.96$, significant at the 99.9% level. The relationship between δD and $\delta^{18}\text{O}$ of rocks or minerals that form through interaction with meteoric fluids at a given temperature is governed by¹⁵

$$\delta\text{D} = 8 \frac{\alpha(\text{H})}{\alpha(\text{O})} \delta^{18}\text{O} + 1,000 \left(8 \frac{\alpha(\text{H})}{\alpha(\text{O})} - 6.99 \alpha(\text{H}) - 1 \right) \quad (1)$$

where $\alpha(\text{H})$, $\alpha(\text{O})$ are the appropriate mineral/water isotope

fractionation factors for hydrogen and oxygen. In general $\alpha(\text{H}) < 1$, whereas $\alpha(\text{O}) > 1$, so the slope is slightly <8. A second conclusion, therefore, is that the fluids determining the agate stable isotope ratios had at least a component of meteoric origin. For Devonian samples there is insufficient resolution to distinguish between meteoric waters from different parts of the MWL and mixtures of meteoric water with seawater.

Different temperatures of isotope exchange between agates and their formation fluids will produce a displacement in the $\delta\text{D}/\delta^{18}\text{O}$ diagram, manifest as a variation in the line intercept (see ref. 15). The data suggest either that Lower Devonian and Tertiary agates from Scotland both formed at the same temperature from waters on the same meteoric water line, or that different water lines and temperatures were involved and their respective effects cancelled. The former seems more plausible. According to the Knauth-Epstein⁹ empirical calibration for chert $\delta^{18}\text{O}$, the temperature is ~46 °C and the rate of change of temperature with difference between water and silicate $\delta^{18}\text{O}$ is 5 °C for each 1‰. Extrapolating the high-temperature absolute quartz/water calibration of Clayton *et al.*¹⁶ gives a temperature of ~63 °C. The hydrogen isotope fractionation between chert MQ and water has been suggested to be between 60 and 80‰ at ocean bottom temperatures¹⁰, and a temperature rate of change of ~1.3‰ °C⁻¹ can be estimated (data from Fig. 8.2 of ref. 15). From the isotopic value of Tertiary meteoric water, we derive an approximation to the hydrogen isotopic fractionation of ~60‰ for the agates. If part of the cause of the spread of Devonian points along the agate line in Fig. 2 is that seawater-meteoric water mixtures were involved, then points high on the line are more likely to have a greater seawater component. It

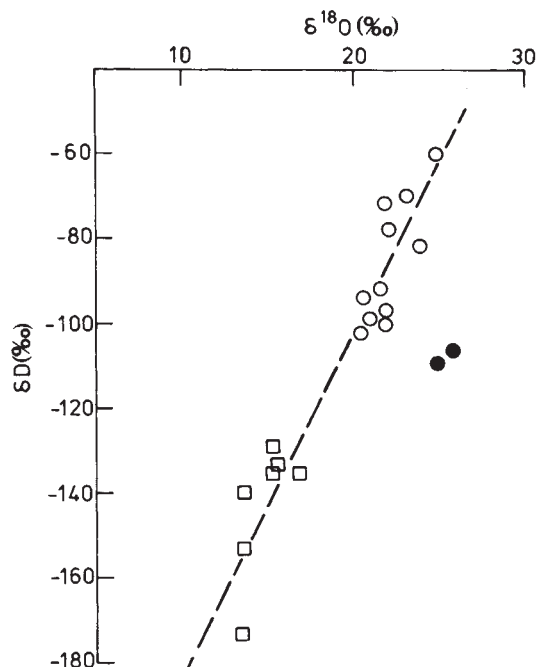


Fig. 2 Plot of δD (relative to V-SMOW) of H_2O^+ against silicate $\delta^{18}O$ (relative to V-SMOW) for Scottish agates. The circles are Lower Devonian samples, with filled symbols for JJ12 and JJ17 (see text). The squares are Tertiary samples. The dotted line is $\delta D = 8.4\delta^{18}O - 271$, the best-fit line through the 11 open circles, shown extrapolated to the region of the Tertiary data.

may be significant that six points plot low, close together at $\sim(+21\%, -98\%)$; these could represent a pure meteoric water endmember. Assuming this and adopting 60‰ for the δD fractionation, Lower Devonian waters would be $\sim(-5\%, -30\%)$.

The high $\delta^{18}O$, low δD samples JJ12 and JJ17 are problematic. We speculate that for some, as yet unknown, reason they formed at a lower temperature than the others. Assuming that the same formation waters were involved, the $\delta^{18}O$ shift implies a temperature of 30 °C (Knauth-Epstein¹⁰ calibration) or 50 °C (Clayton *et al.*¹⁶ calibration). The agate/water δD fractionation of 80‰ would then correspond approximately to a rate of change of 20‰ in 16 °C or $\sim 1.3\% \text{ } ^\circ\text{C}^{-1}$. This is the same as the value derived from ref. 15 and so the argument is at least consistent. Contrary to previous discussions^{1,2,6,8}, our work strongly supports a low-temperature genesis for agates, and similar temperature estimates for the majority of Lower Devonian and Tertiary agates are a necessary consequence of our interpretation of the data distribution as representing a co-linear trend parallel to a common meteoric water line.

Returning to the idea of agates as 'refused' marine chert xenoliths, we note that δD of deep-sea cherts is not a function of $\delta^{18}O$ (ref. 11). Also, the data in Fig. 2 show convincingly for the Scottish samples that meteoric water had a role in establishing the isotopic compositions. May the arguments² be relaxed, then, to allow some agates to be refused freshwater cherts (see ref. 17)? This seems unlikely. The investigation⁹ of nodular and bedded cherts suggested $\delta D = 8\delta^{18}O - 328$ for Tertiary samples and $\delta D = 8\delta^{18}O - 279$ for Devonian samples from continental North America, yet a notable feature of the data in Fig. 2 is that a single line is defined for both Devonian and Tertiary agates, the majority of points plotting close to $\delta D = 7.9\delta^{18}O - 260$. Tectonic difficulties associated with the xenolithic idea are exacerbated by the complication that although $\delta^{18}O$ of deep-sea cherts might not be finally established until ~ 10 Myr after deposition¹¹, the agates found in Scottish Tertiary lavas appear to have interacted with highly depleted—and by implication Tertiary—waters during formation.

The relationship between $\delta^{18}O$ of microcrystalline quartz and δD of bound water implies that formation fluid has been retained. Models of agate genesis that appear to involve

sequences of fluids^{1,18} can now be tested isotopically. For other agate suites, like those discussed here, it is possible to estimate the isotopic composition of the local meteoric water during past geological times.

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1. Florke, O. W., Kohler-Herbertz, B., Langer, K. & Tonges, I. *Contr. Miner. Petrol.* **80**, 324–333 (1982).
2. Blankenburg, H.-J., Pilot, J. & Werner, C.-D. *Chem. Erde* **41**, 213–217 (1982).
3. Blankenburg, H.-J., Werner, C.-D., Schron, W. & Klemm, W. *Z. geol. Wiss.* **10**, 1287–1298 (1982).
4. Braitsch, O. *Heidelb. Beitr. Miner. Petrogr.* **5**, 331–372 (1957).
5. Frondel, C. *The System of Mineralogy* (Vol. III) *Silica Minerals* (Wiley, London, 1962).
6. Blankenburg, H.-J. & Berger, H. *Chem. Erde* **40**, 139–145 (1981).
7. Blankenburg, H.-J., Schron, W., Starke, R. & Klemm, W. *Chem. Erde* **42**, 157–172 (1983).
8. Blankenburg, H.-J. *Jena Rev.* **2**, 68–70 (1983).
9. Knauth, L. P. & Epstein, S. *Geochim. cosmochim. Acta* **40**, 1095–1108 (1976).
10. Knauth, L. P. & Epstein, S. *Earth planet. Sci. Lett.* **25**, 1–10 (1975).
11. Kolodny, Y. & Epstein, S. *Geochim. cosmochim. Acta* **40**, 1195–1209 (1976).
12. Taylor, H. P. & Forester, R. W. *J. Petrol.* **12**, 465–497 (1971).
13. Forester, R. W. & Taylor, H. P. *Earth planet. Sci. Lett.* **32**, 11–17 (1976).
14. Forester, R. W. & Taylor, H. P. *Am. J. Sci.* **277**, 136–177 (1977).
15. Savin, S. M. in *Handbook of Environmental Isotope Geochemistry* Vol. 1 (eds Fritz, P. & Fontes, J. Ch.) 283–327 (Elsevier, Amsterdam, 1980).
16. Clayton, R. N., O'Neil, J. R. & Mayeda, T. K. *J. geophys. Res.* **77**, 3057–3067 (1972).
17. Knauth, L. P. *Geology* **7**, 274–277 (1979).
18. Sunagawa, I. & Ohta, E. *Sci. Rep. Tohoku Univ.* **13**, 131–146 (1976).

Redistribution of fallout radionuclides in Enewetak Atoll lagoon sediments by callianassid bioturbation

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The lagoon sediments of Enewetak Atoll in the Marshall Islands contain a large selection of fallout radionuclides as a result of 43 nuclear weapon tests conducted there between 1948 and 1958 (refs 1, 2). Studies of the burial of fallout radionuclides have been conducted on the islands^{3,4} and in several of the large craters^{1,5}, but studies of their vertical distribution have been limited to about the upper 20 cm of the lagoon sediments. We have found elevated fallout radionuclide concentrations buried more deeply in the lagoon sediments and evidence of burrowing into the sediment by several species of callianassid ghost shrimp (Crustacea: Thalassinidea) which has displaced highly radioactive sediment. The burrowing activities of callianassids, which are ubiquitous on the lagoon floor, facilitate radionuclide redistribution and complicate the fallout radionuclide inventory of the lagoon.

The Enewetak radiological survey of 1972 (ref. 1) extensively sampled the surface sediment of the atoll lagoon and identified

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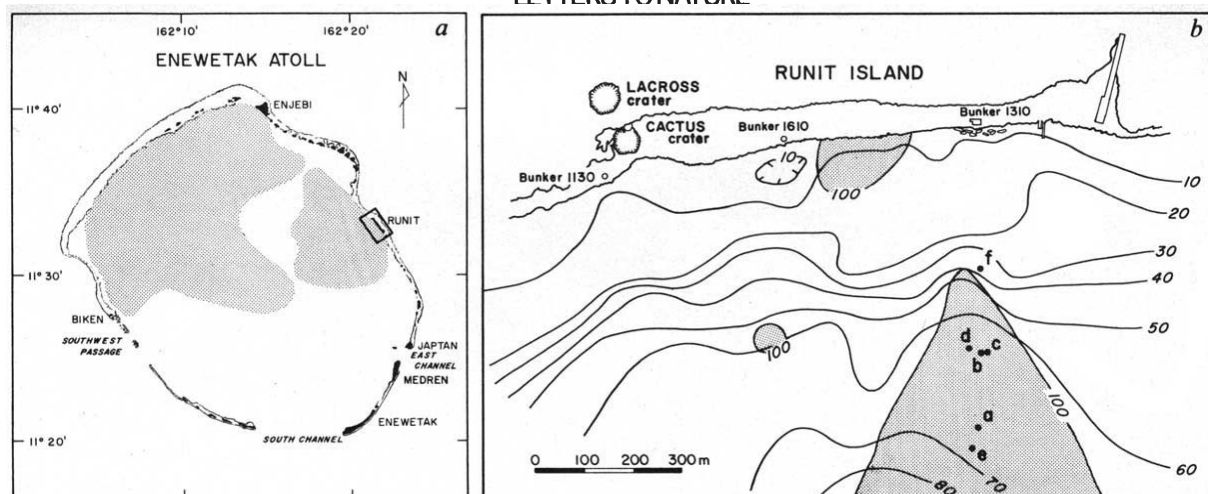


Fig. 1 a, Location map of Enewetak Atoll, Marshall Islands. Shaded areas are surficial lagoon sediments with $>100 \text{ mCi km}^{-2} \text{ }^{239+240}\text{Pu}$. Box indicates location of detailed study area off Runit Island (after ref. 1). b, Detailed study area showing sample locations a-f (G.M.M. et al., unpublished data). Shaded areas are surficial lagoon sediments with $>100 \text{ pCi g}^{-1} \text{ }^{239+240}\text{Pu}$. Bathymetric contours in feet (after ref. 1).

two areas having significant contamination: the north-west lagoon extending south-west of Enjebi and a smaller area extending south-west of Runit (Fig. 1a). Because these sediments contain most of the atoll radionuclide inventory and possess an active, burrowing infauna, we investigated the vertical distribution of fallout radionuclides in a series of cores taken by SCUBA divers within the contaminated area south-west of Runit Island (Fig. 1b).

Eleven sediment cores between 20 and 200 cm in length were recovered from an area of the lagoon floor suspected to contain the fallout contamination of several nuclear detonations on floating barges (Fig. 1b). The cores were recovered by hammering 3-inch diameter plastic core liners into the sediment, which were capped and extracted. Six of these cores exhibit large amounts of radioactivity in distinct layers as identified by γ spectrometry, α spectrometry, and α and β autoradiographic techniques. Two of these cores, 5C-1 and 5D-3 (sites c and d in Fig. 1b), were singled out for the present detailed analyses because: (1) they contained unusually large amounts of radioactivity associated with a thick distinct layer of fine-grained carbonate material and (2) they readily displayed the effects of bioturbation. A more comprehensive report on these sediments will appear elsewhere (G.M.M. et al., unpublished data).

The lagoon sediments are primarily composed of an unconsolidated mixture of the skeletal remains of corals, coralline algae (*Halimeda* sp.), and foraminifera⁶. The lagoon floor in the sampling area is populated by scattered *Halimeda* colonies and ubiquitous mounds of reworked sediment and associated 'sink holes' produced by populations of several species of burrowing callianassid ghost shrimp (Crustacea: Thalassinidea). At

least four different species have been identified, each in a separate genus and all from the family Callianassidae (*Callianassa* sp., *Calliax* aff. *C. novaebritanniae* (Borradaile), *Callichirus vigilax* (de Man) and *Thomassinia* sp.). Numerous other invertebrates and fishes at Enewetak contribute to bioturbation of lagoon sediments, but callianassid shrimps seem to have the greatest impact on sediment redistribution. During feeding and tunnel construction these callianassids often burrow to depths of $>1.5 \text{ m}$ below the sediment surface and sort through massive quantities of sediment to glean organic material⁷. Coarse-grained sediments ($\geq 1.4 \text{ mm}$) are stored in subsurface refuse galleries. Smaller particles are either used in burrow wall construction or pumped to the sediment-water interface where they are either suspended in the water column or accumulate in the form of mounds^{7,8}. The two cores described here were taken 60 m apart among dense callianassid mounds; both cores display evidence of callianassid burrowing (Figs 2 and 3).

The sediment cores were split lengthwise into two halves. One-half was surveyed by a scanning γ -spectrometer system equipped with a NaI(Tl) detector. The remaining half was surveyed by α and β autoradiography. α -Sensitive, Kodak LR-115 cellulose nitrate film was placed directly on the core for 2 months and developed as described in ref. 9. α Tracks were counted by a random field-of-view method with a light microscope. To assess total β activity, Kodak AA-2 Industrial X-ray film was placed on the core half for 4 months. A 0.01 mm thick plastic sheet was placed between the core and X-ray film to prevent chemical reactions and α -particle exposure. High-energy γ rays are too energetic to expose this film. The films were scanned with a densitometer and generated density plots

Table 1 Radionuclide concentrations in Enewetak Lagoon sediments off Runit Island (pCi g^{-1})

Core no.	Depth (cm)	^{60}Co	^{125}Sb	^{137}Cs	^{152}Eu	^{154}Eu	^{155}Eu	^{207}Bi	^{226}Ra	^{238}Pu	$^{239+240}\text{Pu}$	^{241}Am
5C-1	0-5	35 $\pm$ 1	1.6 $\pm$ 0.2	11.1 $\pm$ 0.3	0.7 $\pm$ 0.1	0.3 $\pm$ 0.1	35 $\pm$ 1	0.6 $\pm$ 0.1	0.1†	(12 $\pm$ 2)	(130 $\pm$ 20)	19 $\pm$ 1
	0-5*	23 $\pm$ 1	0.9 $\pm$ 0.1	8.2 $\pm$ 0.1	0.7 $\pm$ 0.1	0.1	30 $\pm$ 1	0.5 $\pm$ 0.1	0.1†	8.6 $\pm$ 0.1	101 $\pm$ 1	17 $\pm$ 2
	5-9	28 $\pm$ 1	1.4 $\pm$ 0.1	10.1 $\pm$ 0.2	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1	27 $\pm$ 1	0.5 $\pm$ 0.1	0.1†	9.4 $\pm$ 0.1	108 $\pm$ 1	15 $\pm$ 2
	48-53	445 $\pm$ 4	46 $\pm$ 1	101 $\pm$ 2	2.3 $\pm$ 0.5	0.6	132 $\pm$ 2	0.2	0.4†	(6 $\pm$ 1)	(63 $\pm$ 9)	9 $\pm$ 1
5D-3	133-138	35 $\pm$ 1	3.6 $\pm$ 0.2	59 $\pm$ 1	0.1	0.2	13.1 $\pm$ 0.3	0.1	0.1†	(0.7 $\pm$ 0.1)	(8 $\pm$ 1)	1.2 $\pm$ 0.3
	0-5	14 $\pm$ 1	0.5 $\pm$ 0.1	2.1 $\pm$ 0.1	0.4 $\pm$ 0.1	0.1	21.5 $\pm$ 0.4	1.6 $\pm$ 0.1	0.1†	(14 $\pm$ 2)	(150 $\pm$ 20)	22 $\pm$ 1
	5-8	11.9 $\pm$ 0.1	0.5 $\pm$ 0.1	1.9 $\pm$ 0.1	0.4 $\pm$ 0.1	0.1	19.4 $\pm$ 0.3	1.4 $\pm$ 0.1	0.3 $\pm$ 0.1	12.5 $\pm$ 0.1	114 $\pm$ 1	19 $\pm$ 1
	32-36	47 $\pm$ 1	3.5 $\pm$ 0.2	3.7 $\pm$ 0.2	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1	30 $\pm$ 1	3.7 $\pm$ 0.1	0.4 $\pm$ 0.1	26.7 $\pm$ 0.3	295 $\pm$ 3	37 $\pm$ 2
	61.5-66.5	1424 $\pm$ 7	149 $\pm$ 1	166 $\pm$ 3	8 $\pm$ 1	4.2 $\pm$ 0.7	405 $\pm$ 8	0.4	0.6†	(36 $\pm$ 5)	(360 $\pm$ 50)	55 $\pm$ 5
	109-113	62 $\pm$ 2	5.4 $\pm$ 0.2	5.4 $\pm$ 0.2	0.6 $\pm$ 0.1	0.2	21.7 $\pm$ 0.4	0.1	0.1†	(1.0 $\pm$ 0.1)	(11 $\pm$ 1)	1.6 $\pm$ 0.4

Analyses performed at Lawrence Livermore Laboratory by γ spectrometry using a Ge(Li) detector except Pu analyses by α spectrometry. Errors are based on counting statistics ($\pm 1\sigma$). Bracketed Pu values are estimated from mean Pu/Am isotope ratios.

* Replicate analysis based on adjacent sample of core. All analyses reported as of 5 March 1982.

† Upper limit values only.

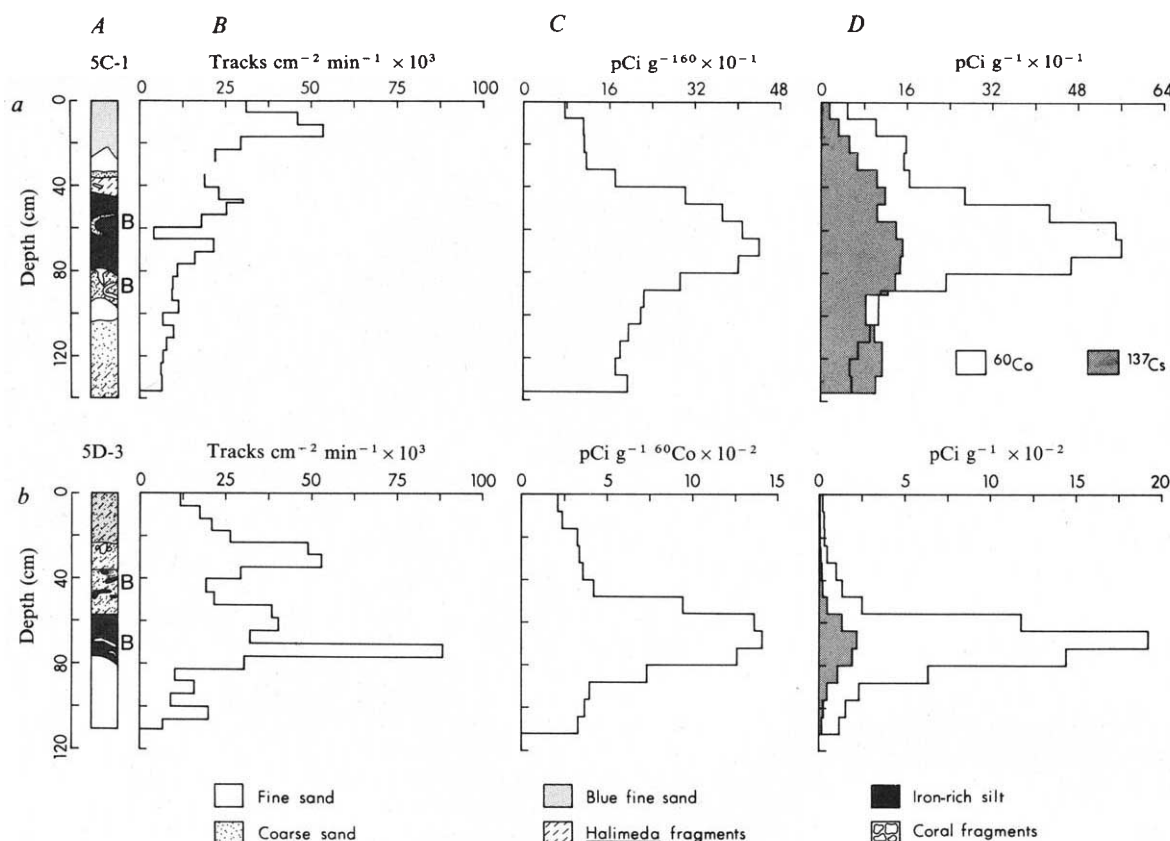


Fig. 2 Detailed vertical radioactivity distributions in cores Runit 5C-1 (a) and 5D-3 (b). A, General lithological descriptions of sediment cores. Major areas of burrows are indicated by 'B'. B, Total α -activity distribution. C, Total β -activity distribution as ^{60}Co . D, Distribution of ^{60}Co and ^{137}Cs .

were integrated by using a polar planimeter. Total β activities were standardized with a ^{60}Co source designed to approximate the self-absorption of the cores.

Selected samples of the cores were analysed by α and γ spectrometry at Lawrence Livermore Laboratory. Samples for γ analysis were dried and coarsely ground before counting on a Ge(Li) spectrometer system. Splits of these samples were dissolved in concentrated acids and Pu was preconcentrated by anion exchange before α spectrometry using Si(Li) detectors.

These sediments contain a complex assemblage of nuclear fuel, fission and activation products and their decay products^{1,2}. The abundance of many of the long-lived radionuclides that contribute to the α , β and γ activity are presented for selected sections of the cores in Table 1. Detailed vertical radioactivity distributions of total α , total β , and the major β and γ -emitting isotopes ^{60}Co and ^{137}Cs are shown in Fig. 2.

Both cores contain discrete layers of predominantly fine-grained ($<38\ \mu\text{m}$) carbonate material which correspond to peaks in radioactivity at depths of 43–80 cm and 56–88 cm, respectively, for cores 5C-1 and 5D-3 (Fig. 2). This fine-grained carbonate is probably blast-pulverized sediment and reef material formed by cratering from nearby barge events. After an almost instantaneous deposition of the fine-grained carbonate layer, deposition of predominantly coarser-grained sediments occurred by either slumping, normal sedimentation or some combination of these processes (Fig. 2). Similar fine-grained sediments have been observed in and adjacent to the Enewetak nuclear craters on land⁵. These barge events occurred during the Hardtack Phase I Series from May to August of 1958, the last series of tests conducted at Runit².

Chemical, mineralogical and scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDAX) of carbonate dissolution residues reveal that these fine carbonate layers contain 1–2 wt % non-carbonate material composed largely of magnetite with minor amounts of pyrite and amorphous biogenic

silica. This noncarbonate material contains $>90\%$ of the total radioactivity in the sample.

Adams *et al.*¹⁰ found that fallout particles collected following nuclear weapons detonations at Enewetak were formed by the interaction of condensing vaporized metals and fission products from the bomb and associated structures with surface material swept up into the cooling fireball. In the case of barge events, these particles were largely small ($<1\ \mu\text{m}$) spheres of dicalcium ferrite formed from the condensation of the vaporized barge and ballast materials; magnetite and calcium oxide were identified as major components of fallout particles from tower and ground shots on land¹⁰.

Probably most, if not all, of the radioactivity in these sediments was originally present in the magnetite. SEM-EDAX results indicate that the pyrite has formed *in situ* by postdepositional interaction of dissolved Fe^{2+} and sulphide derived from bacterially mediated reduction of porewater sulphate in anoxic conditions¹¹. Inspection of the fine-grained carbonate layers both immediately after collection of the cores and after splitting revealed black and greyish-brown colours for 5D-3 and 5C-1, respectively, indicating that core 5C-1 was oxidized relative to 5D-3. X-ray diffraction analysis of the noncarbonate fraction of both cores indicates the presence of X-ray amorphous material in the 5C-1 layer that may be iron (III) oxide; chemical analysis of both fractions rule out the possibility that this amorphous material is composed of a greater amount of biogenic silica.

Because of their similar lithological, chemical, mineralogical, and radionuclide compositions, it seems likely that the two closely spaced cores have sampled either the same radioactive layer or layers produced by closely spaced tests of similar effect. Visual observation of both cores suggests that core 5C-1 is more heavily bioturbated than 5D-3. Even in core 5D-3, β autoradiography reveals that burrowing into the fine carbonate layer has displaced highly radioactive material and replaced it with less radioactive coarser-grained material from layers above

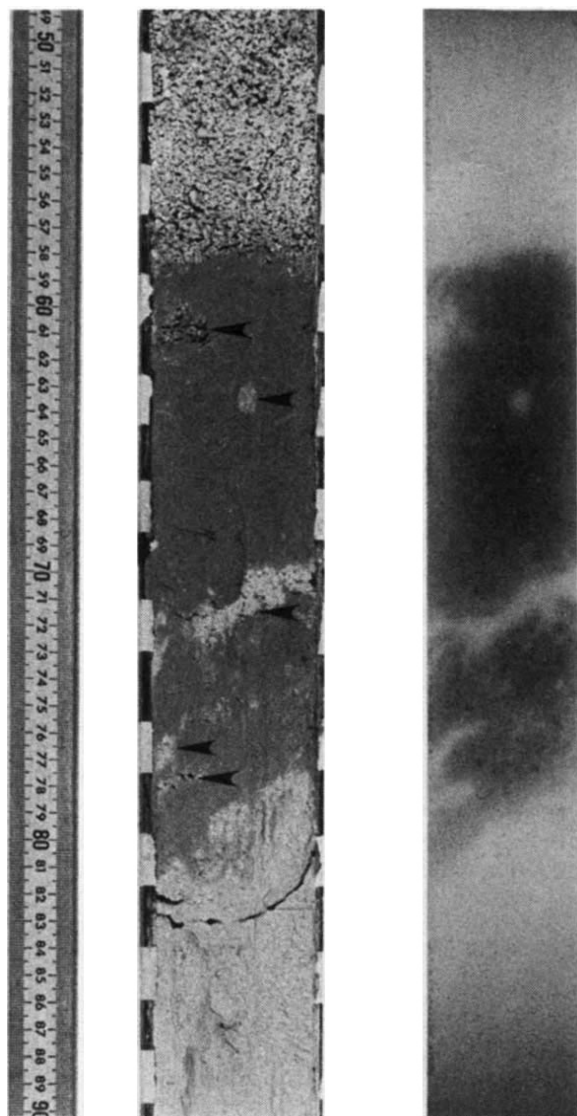


Fig. 3 Iron-rich, carbonate silt layer in core 5D-3. Callianassid burrows are indicated by arrowheads. β autoradiograph (right) shows callianassid burrows filled with less radioactive sediment from adjacent layers (light tones). Dark tones indicate highly radioactive sediment.

and below the fine carbonate (Fig. 3). The fine carbonate layer of core 5C-1 contains about one-third of the iron content and overall radioactivity of core 5D-3 (Table 1 and Fig. 2). Comparison of grain-size distributions in samples taken from these layers where no major burrows are evident suggests that the 5C-1 layer also contains a greater amount of coarser-grained carbonate material.

These observations suggest greater mixing of less-radioactive material into or greater transport of more-radioactive material out of the 5C-1 fine carbonate layer, or both. Callianassid burrowing could easily account for any combination of these sediment transport processes. The relative degree of burrowing into these layers may also be reflected by their relative degree of oxidation. For at least the absolute concentrations, however, we cannot rule out core-to-core variations over small distances due to such factors as variable deposition or even variable test yield should the cores have sampled different blast areas. Indeed, the different radionuclide ratios in the fine-grained carbonate layers of the two cores, if unaffected by the bioturbation, strongly suggest that they have received debris from different tests (Table 1).

The distributions of ^{60}Co , ^{137}Cs and transuranic activities suggest either different deposition or redistribution of

transuranic isotopes relative to that of ^{60}Co in both cores and of ^{137}Cs relative to ^{60}Co in core 5C-1 (Table 1 and Fig. 2). Redistribution, by callianassid activity or another process, assumes that most of the radioactivity observed in the two cores originated in the fine carbonate layers and that no significant radioactive deposition occurred in the area before or after the deposition event. It is unfortunate that, given the complex history of testing at Enewetak Atoll and, in particular, the lagoon area off Runit Island^{1,2}, deposition of ^{137}Cs and transuranic isotopes before and after those deposited in the fine carbonate layers cannot be excluded.

Our findings have implications for both the atoll radionuclide inventory and for the mobilization of this inventory in the sediments and bottom waters of the lagoon. Previous estimates of the inventory identified the lagoon sediments as the largest reservoir, but relied on samples within the topmost 20 cm of the sediment column^{1,12}. These estimates are certainly minimum values. Although our samples are from a small portion of the lagoon where burial of radionuclides may only be associated with cratering, it is precisely those areas where the levels of contamination are greatest¹. If we use this area to extrapolate the lagoon sediment radionuclide inventory, previous estimates are 3–10 times too low. Further, a significant portion of the initial surface fallout deposition to the entire lagoon floor may be mixed to depths of at least 1.5 m by callianassid activity. Burial of fallout radionuclides by the bioturbation of unspecified benthic burrowing organisms is often invoked to explain the vertical distribution of these radionuclides in the topmost 10–30 cm of marine sediments^{13–15}, and the vigour and scale of burrowing activity in the marine environment has become more widely recognized^{7,8,16–20}. Callianassids are found at all water depths within the lagoon; the mean number of large (≥ 5 cm diameter) mounds increases from 1 m^{-2} at 15–30 m to 2.5 m^{-2} below 30 m and sometimes exceeds 7 m^{-2} . Deep-sediment mixing by callianassids would, therefore, add to the underestimation of lagoon sediment radionuclide inventories based on values determined from surface samples taken 14 yr after the testing period. Where fallout radionuclides have been deeply buried, the burrowing abilities of callianassids may recycle radionuclides to the sediment–water interface where dispersion into the overlying water column would be enhanced.

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1. US Atomic Energy Commission, *Enewetak Radiological Survey* NVO-140, Vol. 1 (Nevada Operations Office, Las Vegas, 1973).
2. US Department of Energy, *Enewetak Radiological Support Project* NVO-213, Final Rep. (Nevada Operations Office, Las Vegas, 1982).
3. Gudiksen, P. H. & Lynch, O. D. T. *Jr Health Phys.* **29**, 17–25 (1975).
4. Bliss, W. (compiler), *Enewetak Fact Book* (US Department of Energy, Nevada Operations Office, Las Vegas, 1982).
5. Ristvet, B. L. *Geological and Geophysical Investigations of the Enewetak Nuclear Craters* AFWL-TR-77-242 (Air Force Weapons Laboratory, Air Force Systems Command, Kirtland AFB, 1978).
6. Emery, K. O., Tracy, J. I. Jr & Ladd, H. S. *Geol. Surv. Prof. Pap.* 260-A (1954).
7. Suchanek, T. H. *J. mar. Res.* **41**, 281–298 (1983).
8. Roberts, H. H., Suchanek, T. H. & Wiseman, W. J. *Proc. 4th int. Coral Reef Symp.*, Manila, **1**, 459–465 (1982).
9. Buddemeier, R. W., Biermann, A. H. & Gatrousis, C. *Lawrence Livermore Laboratory Rep.* UCRL-80679 (1978).
10. Adams, C. E., Farlow, N. H. & Schell, W. R. *Geochim. cosmochim. Acta* **18**, 42–56 (1960).
11. Berner, R. A. *Am. J. Sci.* **268**, 1–23 (1970).
12. Noshkin, V. E. in *Transuranic Elements in the Environment* (ed. Hanson, W. C.) 578–601 (US Department of Energy, Washington DC, 1980).
13. Peng, T.-H., Broecker, W. S., Kipphut, G. & Shackleton, N. in *The Fate of Fossil Fuel CO₂ in the Oceans* (eds Anderson, N. R. & Malahoff, A.) 355–373 (Plenum, New York, 1977).
14. Livingston, H. D. & Bowen, V. T. *Earth planet. Sci. Lett.* **43**, 29–45 (1979).
15. Santachi, P. H. *et al. Geochim. cosmochim. Acta* **47**, 201–210 (1983).
16. Shinn, E. A. *J. Paleont.* **42**, 879–894 (1968).
17. Ott, J. A., Fuchs, B., Fuchs, R. & Malasek, A. *Senckenbergiana Maritima* **8**, 61–79 (1976).
18. Pemberton, G. S., Risk, M. J. & Buckley, D. E. *Science* **192**, 790–791 (1976).
19. Weaver, P. P. E. & Schultheiss, P. J. *Nature* **301**, 329–331 (1983).
20. Kershaw, P. J., Swift, D. J., Pentreath, R. J. & Lovett, M. B. *Nature* **306**, 774–775 (1983).

Independence of the circadian rhythm in alertness from the sleep/wake cycle

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It is common knowledge that our feelings of alertness or drowsiness vary throughout the day. Indeed, this diurnal variation is so widely accepted that it has been used to validate the drowsy/alert component of activation obtained from mood adjective checklists¹. There is, however, some evidence from sleep deprivation² and shiftwork³ studies that this variation is not simply a reflection of our sleep/wake cycle, as might be expected, but is at least partially dependent on an endogenous circadian (~24 h) oscillator such as that proposed to account for the circadian rhythm in body temperature and other physiological variables. Here we have tested this suggestion by separating the body-temperature rhythm from the sleep/wake cycle by progressively shortening artificial time cues (zeitgebers)⁴. Our results indicate that the circadian rhythm in alertness can become independent of both the sleep/wake cycle and the rhythm in body temperature. Further, and contrary to our expectations, the results suggest that the sleep/wake cycle exerts less influence on the alertness rhythm than it does on that of temperature.

Current models of the control of circadian rhythms and the sleep/wake cycle typically assume two main underlying processes: the first is a strong endogenous circadian oscillator that is relatively impervious to exogenous influences⁵⁻⁷ and can be entrained to a narrow range of periods only; the second process is generally held to be weaker in that it is more susceptible to exogenous influences and can be entrained to a larger range of periods⁵⁻⁷. These two processes interact to determine our overt circadian rhythms. Some rhythms, however, such as those of temperature and urinary potassium excretion, are believed to be controlled dominantly by the strong endogenous oscillator, while the second process is thought to be dominant in controlling the sleep/wake cycle and, for example, the circadian rhythm in urinary phosphate excretion. Evidence that these processes interact is provided by the dependence of sleep duration and structure on the phase of the temperature rhythm at which sleep onset occurs^{8,9}.

Under normal circumstances both processes will be entrained to a period of 24 h by the zeitgebers in our environment. However, under the abnormal sleep/wake routines resulting from shiftwork, rapid time-zone transitions, prolonged temporal isolation and prolonged sleep deprivation, their separate influences may be manifested¹⁰. Examination of rhythms of subjective alertness in such conditions suggests, somewhat counter-intuitively, some independence from the sleep/wake cycle^{2,3}. We have used Wever's fractional desynchronization technique^{11,12} to explore the relative dependence of the alertness rhythm on the endogenous circadian oscillator controlling temperature and on the sleep/wake cycle. This technique involves progressively changing the period of artificial zeitgebers beyond the limit of entrainment of the strong endogenous oscillator. Rhythms dominantly controlled by this oscillator thus 'break out' from the zeitgebers and free-run with the endogenous period of this oscillator, while those dominantly controlled by the weaker process, such as the sleep/wake cycle, remain entrained for longer.

Twelve apparently healthy student volunteers (aged 18-21 yr) were isolated, in three groups (two male groups, 1 female) of four, from all normal zeitgebers for 3 weeks in a temporal isolation unit¹³. They were exposed to local zeitgebers produced

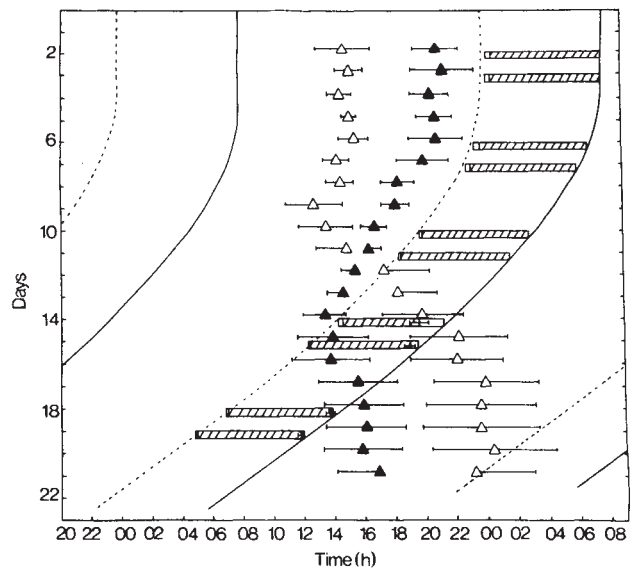


Fig. 1 The mean ($\pm$ s.e.) estimates of the peak (or 'acrophase') of the temperature ($\blacktriangle$) and alertness ($\triangle$) rhythms on successive 'days'. The curved lines show the real timing of local '23.45' (---) and '07.45' (—). The bars between these lines show the timing of the polygraphically recorded sleep periods (open = awake; shaded = asleep). The standard errors of sleep onset and end are also shown where these were large enough to be represented.

by a visible analog clock controlled by a microprocessor. For the first four complete days of the study the clock ran at the correct rate. After this, and unknown to the subjects, it ran faster by 0.2 h each 'day' down to a 'day' of 23.0 h, and then by 0.1 h each 'day' until a 'day' of 22.0 h was reached. It then ran at a constant rate of 22.0 h per 'day' for the remainder of the study (four complete 'days'). Changes in the rate of the clock were made at 'local' midnight. Throughout the experiment, and always according to this 'local' clock, the subjects were requested to take their meals at a constant time of 'day' and to retire to bed at 23.45, and they were woken by an alarm at 07.45.

During the study, rectal temperature was measured automatically every 2 min using Vitalog TML2 recorders¹⁴ and subjective ratings of 'drowsiness/alertness' were made on a visual analog scale every 2 h 40 min from 08.00 to 21.20 (local time). In addition, the sleep of eight of the subjects (1 male group and 1 female group) was recorded polygraphically on a 2 'nights' on/2 'nights' off basis throughout the study. The polygraphic sleep records were scored by eye and the detailed results will be reported in full elsewhere. Here we report only whether or not the subject was asleep.

To estimate the 'peak' (or 'acrophase') of each subject's rhythms in alertness and temperature, 24-h sinusoids were fitted to 3-'day' intervals of data incremented by 1-'day' windows¹⁵. In the case of temperature, only those readings corresponding in time to the alertness ratings were included in order to ensure strict comparability of the results and to avoid problems associated with the masking of temperature by sleep^{5,10}. Analysis of the 2-min temperature readings yielded a qualitatively similar pattern of findings⁴.

Figure 1 shows the estimates of mean acrophase for the circadian rhythms in temperature and alertness on successive 'days', together with the times of recorded sleep. For the first 9 'days' of the study (that is, down to a 'day' length of 23.0 h) all the variables followed the artificial zeitgebers very well, and the normal phase relationship between the alertness and temperature rhythms was observed^{1,16}. Subsequently ('days' 10-14), the phase of the alertness rhythm delayed fairly rapidly relative to both the temperature rhythm and the sleep/wake cycle and ran with a mean period of 25.48 h, while the temperature rhythm showed only a slight phase delay relative to the sleep/wake cycle and ran with a mean period of 23.18 h.

These results indicate clearly both an independence of the alertness rhythm from the sleep/wake cycle and a dissociation of the alertness and temperature rhythms. However, the question remains as to whether the temperature rhythm remained entrained by the zeitgebers for this portion of the study, or whether the slight phase delay observed reflected an independence from them. It seems probable that the former was the case as it is known that the phase angle of an entrained rhythm is affected by the zeitgeber period¹⁷, and the present results are in agreement with this relationship. Further, the mean period observed for the temperature rhythm during this portion of the study was considerably shorter than that expected (24.88 ± 0.19 h)⁵ for a temperature rhythm running independently of the sleep/wake cycle. Thus, it seems that, following the 'break out' of the alertness rhythm on 'day' 10, the temperature rhythm remained entrained by the zeitgebers for a further 5 'days' before it, too, phase-delayed relatively rapidly with respect to them. During the final portion of the study, the free-running rhythms in temperature and alertness appear to have become entrained to one another (temperature mean period = 24.49 h, alertness mean period = 24.47 h) but showed a reversal of their normal phase relationship, with the temperature acrophase occurring ~7.5 h before that for alertness.

Analysis of the limits of entrainment of each individual's rhythms indicated that the rhythms of one subject remained entrained for the duration of the study (excluding this subject resulted in an essentially similar pattern of results to that shown in Fig. 1, but with substantially reduced standard errors following the break out of the rhythms). The remaining subjects showed a limit of entrainment of their temperature rhythms (22.48 ± 0.09 h) very similar to previous results¹¹, and which was consistently lower than that of their alertness rhythms (23.00 ± 0.19 h; $t = 7.874$, $P < 0.001$). However, there was no evidence of a relationship between the limits of entrainment of the two rhythms ($r = -0.059$).

These results indicate that the sleep/wake cycle exerts even less influence on the circadian rhythm of alertness than it does on that of body temperature; in terms of current models of the circadian system, this implies either that the circadian rhythm in alertness is a purer reflection of the strong endogenous oscillator than is temperature itself, or that it is controlled by an oscillator separate from that responsible for the temperature rhythm. The failure to find a correlation between the limits of entrainment of the two rhythms favours the latter interpretation as individual differences in the limits of entrainment of a single oscillator would give a correlation. If the rhythm in alertness is indeed controlled by a separate oscillator, it should be possible to induce desynchronization between the temperature and alertness rhythms, rather than the temporary dissociation observed in the present study, by providing zeitgebers with a period between the limits of entrainment of the temperature and alertness rhythms. We intend to perform such a study.

It is clear from the present results that, whether or not the rhythms in alertness and temperature prove to be controlled by separate oscillators, the alertness rhythm can run independently of the sleep/wake cycle, and can become dissociated from the temperature rhythm. Thus, the adjustment of the temperature rhythm to shiftwork or rapid time-zone transitions (that is, jet-lag) may be a rather poor indicant of the adjustment of an individual's rhythm in alertness; this may have implications for performance efficiency in these conditions. However, while it is known that body temperature may be a poor indicant of some types of performance^{12,18}, the role of alertness in this respect has yet to be established.

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4. Folkard, S., Minors, D. S. & Waterhouse, J. M. *J. Physiol., Lond.* **357**, 341–356 (1984).
5. Wever, R. A. *The Circadian System of Man: Results of Experiments under Temporal Isolation* (Springer, New York, 1979).
6. Kronauer, R. E., Czeisler, C. A., Pilato, S. F., Moore-Ede, M. C. & Weitzman, E. D. *Am. J. Physiol.* **242**, R3–R17 (1982).
7. Borbely, A. A. *Hum. Neurobiol.* **1**, 195–204 (1982).
8. Czeisler, C. A., Weitzman, E. D., Moore-Ede, M. C., Zimmerman, J. C. & Knauer, R. S. *Science* **210**, 1264–1267 (1980).
9. Winfree, A. T. *Nature* **297**, 23–27 (1982).
10. Minors, D. S. & Waterhouse, J. M. *Circadian Rhythms and the Human* (Wright P.S.G., Bristol, 1981).
11. Wever, R. A. *Pflügers Arch. ges. Physiol.* **396**, 128–137 (1983).
12. Folkard, S., Wever, R. A. & Wildgruber, Ch. M. *Nature* **305**, 223–226 (1983).
13. Elliot, A. L., Mills, J. N., Minors, D. S. & Waterhouse, J. M. *J. Physiol., Lond.* **221**, 227–257 (1972).
14. Halberg, E. *et al. Chronobiologia* **8**, 253–271 (1981).
15. Monk, T. H. & Fort, A. *Int. J. Chronobiol.* **8**, 193–224 (1983).
16. Monk, T. H., Leng, V. C., Folkard, S. & Weitzman, E. D. *Chronobiologia* **10**, 49–55 (1982).
17. Wever, R. A. *J. Interdiscipl. Cycle Res.* **3**, 253–265 (1972).
18. Hughes, D. G. & Folkard, S. *Nature* **264**, 232–234 (1976).

Depletion of unilateral striatal dopamine impairs initiation of contralateral actions and not sensory attention

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Although Parkinson's disease has traditionally been considered as a motor disorder^{1,2}, there has been much recent interest in the nature and the neural substrates of parkinsonian dementia^{3,4} and cognitive dysfunction⁴. These disabilities, which can induce visuo-spatial impairment⁵ and visual 'neglect'⁶, may also have a bearing on the controversy about the normal functions of the nigrostriatal dopamine (DA) projection and the basal ganglia^{7–9}. The observations that neurones in both substantia nigra and striatum respond to sensory events in terms of neuronal firing^{10,11} or DA release^{12,13}, also suggest a role for striatum in sensorimotor integration. An important behavioural correlate of this integration is the 'sensorimotor neglect' syndrome¹⁴ in animals with unilateral lesions of the nigrostriatal projection who fail to orient to contralateral sensory events. However, this neglect may arise not from contralateral sensory inattention^{14,15}, but from an inability to express this sensory selection via motor output. We present here two lines of evidence that unilateral striatal DA depletion in the rat does not affect sensory attention to visual signals of reward, but rather impairs the initiation (though not the completion) of contralateral motor acts. These results not only help to clarify the function of the nigrostriatal DA projection, but also show that depletion in this system is linked specifically to a process of response initiation, which may be the fundamental impairment in Parkinson's disease.

In procedure (1), two groups of rats were trained to hold their heads in a central hole until an eccentric visual stimulus was presented to one or other side of the head. The two groups were required to report the presence of the light either by moving their heads towards it (SAME group, $n = 6$) or away from it (OPPOSITE group, $n = 6$), hence interrupting the infrared photocell beam within the appropriate adjacent hole. Thus, it could be determined whether unilateral DA depletion would produce: (1) a response bias (for example, moving the head towards the side of the lesion, regardless of where the stimulus was presented), (2) sensory inattention for a particular side regardless of the direction of the motor response required, or (3) some higher-order deficit in sensorimotor integration, shown, for example, as a deficit in making a contralateral response, but only in response to a contralateral sensory event.

After training, each rat received an infusion of 6-hydroxydopamine (6-OHDA) into the mid-ventral head of the caudate nucleus randomly on either the right or left side (see Table 1).

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1. Thayer, R. E. *Motivation Emotion* **2**, 1–34 (1978).
2. Froberg, J. E. *Biol. Psychol.* **5**, 119–134 (1977).
3. Folkard, S., Monk, T. H. & Lobban, M. C. *Ergonomics* **21**, 785–799 (1978).

Table 1 Effects of unilateral 6-OHDA lesion of mid-ventral caudate on regional dopamine concentration (ng per mg tissue wet weight)

	Side	Nucleus accumbens septi	Anterior caudate	Posterior caudate
Procedure (1)	Intact	5.625 ± 0.531	7.492 ± 0.796	12.813 ± 0.519
	6-OHDA-lesioned	3.879 ± 0.521*	2.104 ± 0.378†	2.491 ± 0.463†
	% Decrease	31	72	81
Procedure (2)	Intact	3.784 ± 0.504	7.419 ± 1.447	13.101 ± 0.920
	6-OHDA-lesioned	2.345 ± 0.407	1.707 ± 0.417†	1.656 ± 0.409†
	% Decrease	38	77	87

Male hooded Lister rats housed in standard conditions were maintained at 85% of free-feeding weight (300–350 g), with water freely available. After training, all rats were anaesthetized with chloral hydrate/sodium pentobarbitone (3.0 ml per kg) and, 30 min after pargyline pretreatment (50 mg per kg intraperitoneal), were injected unilaterally with 2 µl of 6-OHDA (4 µg per µl base in 0.1% ascorbic acid in saline solution) in the left or right striatum over a 4-min period via a stainless steel cannula (30 gauge). Stereotactic coordinates were: AP, +2 mm from bregma; L, ±3 mm from midline; V, –5.5 mm from the cortical surface with the incisor bar fixed at +5 mm above the interaural line. At the end of each test procedure (70 days (1) and 27 days (2) after lesion) the animals were killed under ether anaesthesia, and their brains were removed rapidly and dissected on ice²⁴. DA concentrations (given as mean ± s.e.m.) were measured using HPLC with electrochemical detection²⁵. For procedures (1) and (2), assay data were subjected to analysis of variance and pairwise comparisons were made with Student's *t*-test. AP = anterior-posterior, L = lateral, V = vertical.

* $P < 0.05$; † $P < 0.001$.

This treatment was later shown to have produced a substantial depletion of DA with respect to the intact side in the posterior and anterior caudate-putamen, but only small depletions in the nucleus accumbens, a major recipient of 'mesolimbic' DA afferents (Table 1).

After 3 days of recovery, the rats were tested once a day for 6 days. Figure 1 shows that the unilateral lesion produced an alteration in accuracy consistent with a response bias towards the lesioned side. Thus, regardless of where the stimulus occurred, the rats had a significant tendency to respond towards the lesioned side.

Other measures taken during these experiments allow a further specification of the nature of the deficit. We measured the

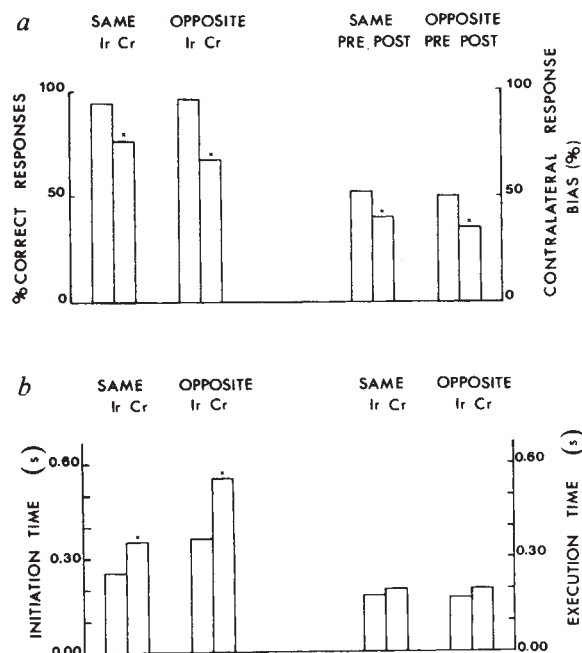
response time (to the nearest 0.01 s) for the rats to make the unilateralized response of withdrawing their head from the central hole following detection of the visual signal, and from this point in time, to make the lateralized response into one of the side holes. These two components of response time were labelled the initiation time and execution (or completion) time, respectively.

There was a significant lengthening of latency for initiation time, but only when the response to be made was contralateral to the side of the lesion (Fig. 1). Thus, this lengthening occurred regardless of the side of presentation of the visual stimulus across the SAME and OPPOSITE groups, and hence cannot reflect a sensory deficit in detecting the visual signal. By contrast,

Fig. 1 Effects of unilateral 6-OHDA lesions of mid-ventral caudate nucleus on accuracy, response bias and on initiation and execution time in procedure (1).

a. The left- and right-hand ordinates show, respectively, the correct responses both ipsi- (Ir) and contralateral (Cr) to the side of the lesion (mean values of six post-operative sessions, days 4–9 inclusive) and the percentage contralateral response bias, for the SAME and OPPOSITE groups (mean values of three sessions preceding and six sessions after surgery). Accuracy was measured as the proportion of correct responses made in each side hole (ipsi- and contralateral) divided by the total number of responses made. Response bias was measured as the proportion of correct plus incorrect responses made into the contralateral hole out of the total number of responses. The analysis of accuracy (% correct) with the factors: GROUP (SAME and OPPOSITE), SIDE (ipsi- and contralateral) and DAYS (post-operative) showed a main effect of SIDE ($F(1,9) = 11.650$, $P < 0.01$) but no interaction of GROUP × SIDE ($F(1,9) = 0.710$). A comparison of pre- and post-operative scores of % response bias using Student's *t*-test showed that the lesion significantly reduced the contralateral response bias ($t(11) = 4.35$, $P < 0.01$). **b.** The left- and right-hand ordinates show, respectively, initiation time (s) and execution time (s) of correct responses ipsi- or contralateral to the side of the lesion for SAME and OPPOSITE groups (mean of six post-operative sessions). Initiation and execution times were measured to the nearest 0.01 s. Initiation and execution time data were log-transformed and subjected to a three-factor analysis of covariance and *post hoc* tests, which showed significantly longer initiation times for contralateral responses for both groups (SAME $t(9) = 2.64$, $P < 0.05$; OPPOSITE $t(9) = 3.5$, $P < 0.01$). There were no significant effects for execution time.

Methods. The visual stimuli were presented on either side of the central hole of a nine-hole array set in the concave, curved wall of an aluminium chamber. Following extensive preliminary training, for both SAME and OPPOSITE groups, the session began by illumination of the house light and the delivery of a free food pellet (45 mg, Abel Co.) behind a hinged Perspex panel located on the wall opposite the array of holes. Opening the panel to collect the pellet turned on the light in the central hole and began the first trial. A sustained nose-poke into the central hole extinguished the light in that hole and started one of four time intervals (0, 0.5, 1.0 and 1.5 s) at the end of which a stimulus light (0.20 s) was presented to either side of the head. Head withdrawal from the central hole extinguished the stimulus light. For the rats in the SAME group a nose-poke response into the illuminated hole resulted in the delivery of food. Responding in the non-illuminated hole resulted in 1 s of darkness (time-out). Conversely, for the rats in the OPPOSITE group, a nose-poke into the non-illuminated hole resulted in the delivery of food, while a response in the illuminated hole was 'punished' by 1 s of darkness. For both groups of rats, responses made at all other times or premature withdrawals of the head from the central hole, before the onset of the stimulus light, were also punished with darkness. Following either the delivery of food or periods of darkness, the rat was required to initiate the next trial by pushing the hinged panel. However, in the cases of premature withdrawal and premature responses, opening the panel after the periods of darkness re-started the current trial. Each daily session consisted of 80 randomly ordered trials, with 10 trials for each spatial location of the stimulus at each time interval.



execution time was unaffected. This dissociation between the two components of response time suggests that the unilateral lesion impaired the processes involved in the initiation of contralateral acts rather than their completion.

A strong test of the hypothesis that unilateral DA lesions produce contralateral deficits in the initiation of motor acts rather than sensory inattention, would be the failure to find neglect in a situation where no lateralized response was required for signal detection. Consequently, in procedure (2), rats were

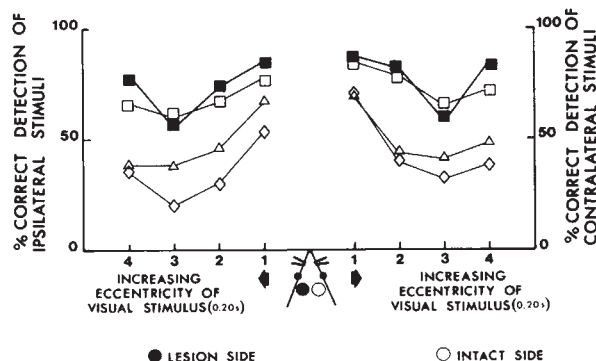


Fig. 2 Effect of 6-OHDA lesions of the mid-ventral caudate nucleus on accuracy of detection of ipsi- and contralateral stimuli of increasing eccentricity in procedure (2). The ordinates show the percentage correct detections for ipsilateral and contralateral stimuli; the abscissa represents increasing eccentricity (1–4) of the visual stimuli. The pre- (■) and post-operative (□) data are mean values of three sessions before surgery and eight (days 4–11 inclusive) after surgery. Data were arc-sine-transformed before analysis of covariance, with factors SIDE (ipsi- and contralateral), DAYS (post-operative) and ECCENTRICITY (1–4). There was a main effect of ECCENTRICITY ($F(3,17) = 6.846$, $P < 0.01$) but no main effects of SIDE ($F(1,5) = 1.847$) or DAYS ($F(7,40) = 1.819$) nor any significant interactions between the three factors. Data from the two test days, DIM1 (Δ) and DIM2 ($\diamond$), were compared with the mean values for two preceding days (control). A three-factor analysis of variance showed a main effect of BRIGHTNESS ($F(2,10) = 19.326$, $P < 0.01$) and ECCENTRICITY ($F(3,15) = 15.869$, $P < 0.01$) but, again, no effect of SIDE or of any of the possible interactions between the three factors.

Methods. We used the same apparatus as for procedure (1) except that while the rat was responding in the central hole, its body was centralized by placing transparent Perspex barriers on either side of its body, ending ~2 cm from the response hole. Each of the other eight holes (four to either side of the central hole) was occluded by a transparent Perspex window, through which visual stimuli were presented. Following extensive preliminary training, the start of the session was signalled by illumination of the house light and the delivery of a free food pellet. Opening of the panel to collect the pellet began the first trial. A discrete nose-poke into the central hole started a variable interval lasting for 12 s on average, after which a stimulus light (0.20 s) on either side of the rat's head was presented behind one of the eight side windows contingent on the first nose-poke after the completion of the variable period. Withdrawal of the nose from the central hole extinguished the stimulus light. A panel push following stimulus presentation, that is, a correct detection, resulted in the delivery of a food pellet. An incorrect nose-poke into the central hole following the presentation of the stimulus was punished by 1 s of darkness. The first panel push occurring after a correct response or after a period of darkness began the next trial. A premature, incorrect panel push before the end of the variable interval was punished by 1 s of darkness. The first panel push following such periods of darkness re-started the current trial. Each daily session consisted of 80 trials (that is, 10 presentations of stimulus light for each of the eight side windows in randomized order) or 30 min of testing, whichever was completed sooner. On two occasions (days 13 and 14 after surgery) the brightness of the visual stimuli was reduced to provide two test conditions, DIM1 and DIM2. The different test conditions were presented to half of the rats in increasing, and to the other half in decreasing, order of brightness.

trained to present brief, eccentric visual stimuli to themselves by poking their noses into the central hole but, in contrast to procedure (1), the seven rats were required to report the visual events by turning around (in either direction) and pushing the Perspex panel located on the opposite wall.

Pre-operative performance was characterized by a gradient of accuracy, with the highest levels attained from the holes nearest to the central hole. Unilateral striatal depletion of DA, using the same method as before (see Table 1), produced no significant changes in the level or gradient of accuracy over a comparable post-operative period to that used for procedure (1) (see Fig. 2), and there were no changes in response latency (the time from the onset of the visual stimulus to a correct panel push). These results show that unilateral striatal DA depletion leads neither to visual inattention on the contralateral side nor to an enhanced sensory attentional bias towards the ipsilateral side.

To test more stringently the sensory inattention hypothesis, we reduced the brightness of the visual stimuli to degrade performance. Although this successfully impaired performance, there was no lateralized effect of the unilateral lesion on accuracy (Fig. 2).

Finally, ~6 weeks post-operatively, in order to demonstrate that the unilateral lesions produced some functional lateralized impairment, we used tests of rotational behaviour induced by 1.5 mg per kg of (+)amphetamine (measured conventionally in white Perspex bowls) and a battery of neurological tests for sensorimotor neglect¹⁶. The results of these tests confirmed that the unilateral lesions had their expected effects. Thus, rotation was induced towards the lesioned side (ipsilateral 233.3 ± 75.2 , contralateral 19.2 ± 7.2 turns min^{-1} ; $t(5) = 2.97$, $P < 0.02$) and the tests of sensory neglect yielded significant deficits in response to events presented to the side contralateral to the lesion (orientation $t(5) = 5.0$, $P < 0.01$; limb use $t(5) = 2.17$, $P < 0.05$; turns on 45° grid $t(5) = 3.8$, $P < 0.01$).

We conclude that visual forms of 'sensory inattention' produced by unilateral DA depletion result from a contralateral impairment of the initiation (and not the execution) of actions directed towards contralateral events. Although these conclusions appear to run counter to those of some other studies of neglect produced by lesions of the basal ganglia^{17,18}, in neither of these studies was the nigrostriatal DA projection selectively destroyed, as has been achieved here. Hence, the other deficits of sensory attention reported previously¹⁸ may be related to damage to non-dopaminergic cells.

Our conclusion that response initiation is impaired following nigrostriatal DA depletion does not exclude an environmental influence on this initiation. Indeed, we have found that the degree of ipsilateral response bias is reduced in procedure (1) if no visual stimuli are presented (M.C. and T.W.R., unpublished results), showing that the response bias is exaggerated by significant environmental events. However, these changes in bias do not result from inattention to stimuli guiding contralateral responses, but probably reflect a nonspecific activational or arousing influence^{19,20}. The present analysis is compatible with electrophysiological experiments showing that cells of the substantia nigra, pars compacta fire predominantly just before or during the execution of reaching movements rather than during the preceding warning signals^{21,22}. Our analysis is also compatible with psychological findings in parkinsonian subjects of smaller gains in speed of reaction time produced by an informative warning stimulus than are found in normal subjects; in normal circumstances the warning signal presumably facilitates response initiation²³. Our results further suggest that nigrostriatal DA depletion in Parkinson's disease contributes mainly to impairments of response initiation. Therefore, the other cognitive dysfunctions of this disease may not be related primarily to nigrostriatal degeneration.

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1. Parkinson, J. *An Essay on the Shaking Palsy* (Whittingham & Bowland, London, 1817).
2. Marsden, C. D. *Neurology* **32**, 514–539 (1982).
3. Mayeux, R. in *Movement Disorders* (eds Marsden, C. D. & Fahn, S.) 75–95 (Butterworth Scientific, London, 1982).
4. Lees, A. J. & Smith, E. *Brain* **106**, 257–270 (1983).
5. Boller, F. et al. *Archs Neurol.* **41**, 485–490 (1984).
6. Villardita, C., Smirni, P. & Zappala, G. *Archs Neurol.* **40**, 737–739 (1983).
7. Marsden, C. D. *Trends Neurosci.* **3**, 284–287 (1980).
8. Oberg, R. G. E. & Divac, I. *Trends Neurosci.* **4**, 122–124 (1981).
9. Cools, A., van den Bercken, J., Hoistink, M., von Spaendonck, K. & Berger, H. *Trends Neurosci.* **4**, 124 (1981).
10. Chiodo, L. A., Caggiula, A. R., Antelman, S. M. & Lineberry, C. G. *Brain Res.* **176**, 385–390 (1979).
11. Steinfels, G. F., Heym, J., Strecker, R. E. & Jacobs, B. L. *Brain Res.* **258**, 217–228 (1983).
12. Hutson, P. H., Knott, P. J. & Curzon, G. in *Central Neurotransmitter Turnover* (eds Pycock, C. J. & Taberner, P. V.) 155–161 (Croom-Helm, London, 1981).
13. Nicoullon, A., Chéramy, A. & Glowinski, J. *Nature* **269**, 340–342 (1977).
14. Marshall, J. F., Richardson, J. S. & Teitelbaum, P. J. *comp. physiol. Psychol.* **87**, 808–830 (1974).
15. Ljungberg, T. & Ungerstedt, U. *Expl Neurol.* **53**, 585–600 (1976).
16. Dunnett, S. B. & Iversen, S. D. *Brain Res.* **248**, 121–127 (1982).
17. Turner, B. H. *J. comp. physiol. Psychol.* **82**, 37–47 (1973).
18. Feeney, D. M. & Weir, C. S. *Brain Res.* **178**, 329–346 (1979).
19. Marshall, J. F., Levitan, D. & Stricker, E. M. *J. comp. physiol. Psychol.* **90**, 536–546 (1976).
20. Wolgin, D. L. & Teitelbaum, P. J. *comp. physiol. Psychol.* **92**, 474–500 (1978).
21. Schultz, W., Ruffin, A. & Achsich, P. *Expl Brain Res.* **51**, 377–387 (1983).
22. Fabre, M., Rolis, E. T., Ashton, J. P. & Williams, G. *Behav Brain Res.* **9**, 213–235 (1983).
23. Bloxham, C. A., Mindel, T. A. & Frith, C. D. *Brain* **107**, 371–384 (1984).
24. Robbins, T. W. & Koob, G. F. *Nature* **285**, 409–412 (1980).
25. Mefford, I. N. *J. Neurosci. Meth.* **3**, 207–224 (1981).

Oxytocin-immunoreactive terminals synapse on oxytocin neurones in the supraoptic nucleus

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The neuropeptide oxytocin, synthesized by magnocellular neurones in the hypothalamus, is well known for its peripheral action during lactation and parturition after its release into the bloodstream from axons in the neurohypophysis. However, it may also be released centrally to control the activity of oxytocinergic neurones themselves. Oxytocin release has been measured from isolated magnocellular nuclei *in vitro*¹ and in the cerebrospinal fluid^{2,3}. When injected into the third ventricle, the peptide increases the basal firing rate of oxytocinergic neurones as well as the frequency and amplitude of the bursts of action potentials they normally show before each reflex milk ejection^{4,5}. Oxytocin also excites magnocellular neurones when applied microiontophoretically⁶. I now report that immunocytochemical staining reveals synapses in the supraoptic nucleus of the hypothalamus where both the pre- and postsynaptic elements contain oxytocin. These oxytocinergic synapses, impinging on their own neurones, may represent the anatomical basis for the hypothalamic release of this peptide and for the facilitatory action on its own secretion.

Tissue from adult male and female Wistar and Brattleboro homozygous (DI) rats was prepared for pre-embedding immunocytochemistry, using the peroxidase/anti-peroxidase technique⁷ as described in detail elsewhere⁸. Briefly, after aldehyde fixation, vibratome sections (50 µm) of regions of the hypothalamus containing the supraoptic nucleus (SON) were incubated in rabbit sera against oxytocin, diluted 1:1,000 (developed by A. Bulet⁹ using synthetic oxytocin glutaraldehyde coupled to thyroglobulin as immunogen) or neurophysin I, diluted 1:2,000 (Paesels; purified bovine neurophysin I served as immunogen).

The immunocytochemical specificity of these antisera was tested in two ways. First, serial aldehyde-fixed vibratome (50 µm) and serial deparaffinized Bouin's fixed (5 µm) sections

through the SON were incubated with each diluted serum, which had been absorbed previously with varying amounts of the following peptides (coupled to CNBR-activated Sepharose 4B according to the manufacturer's manual, Pharmacia): Met-enkephalin, Leu-enkephalin, dynorphin(1–8), β-endorphin, angiotensin (Peninsula, 10–100 µg ml⁻¹ serum), corticotropin releasing factor (CRF), vasoactive intestinal polypeptide (VIP) (0.5 µg ml⁻¹ serum), oxytocin, Arg-vasopressin (Peninsula, 10–100 µg ml⁻¹ serum), neurophysin-I and neurophysin-II (10 µg ml⁻¹ serum). The second test was the incubation of each peptide-coupled Sepharose gel in a test tube with each diluted serum. The sections and gels then underwent the same immunocytochemical staining⁸.

Light microscopy showed that neither serum was crossreactive with any of the first seven peptides, either on the sections (where there was no diminution of staining) or on the gels spread as monolayers (where there was no staining). Staining was completely quenched in sections treated with the sera absorbed with their corresponding peptide. The anti-oxytocin serum did not crossreact with either neurophysin. However, as it showed some crossreactivity with vasopressin, it was used unabsorbed only on tissue from DI rats (genetically deficient in vasopressin) and always preabsorbed with Sepharose-vasopressin (10–50 µg ml⁻¹ diluted serum) for incubation of tissue from normal animals. The anti-neurophysin-I serum showed no crossreactivity to either vasopressin or oxytocin, but was highly crossreactive to neurophysin-II; it was used only on tissue from DI rats. Additional conventional staining controls⁷ on the sections and gels all gave negative results. Note that SON neurones contain no endogenous peroxidase activity¹⁰.

After light microscopic observation, blocks containing the nucleus were selected from the vibratome sections, osmicated and processed as usual for electron microscopy⁸. Blocks derived from control vibratome slices (incubated, for example, with preimmune serum or with serum absorbed with its corresponding peptide) were concomitantly prepared and examined.

In ultrathin sections of nuclei incubated with either anti-oxytocin or anti-neurophysin sera, many neuronal structures contained variable amounts of electron-dense immunoprecipitate; surrounding structures showed no trace of reaction product (Figs 1, 2). Such specific staining was never observed in the control sections. As expected, in the SON of normal or DI rats, most elements immunopositive for oxytocin or neurophysin I were somata and dendrites. However, we also found immunopositive 'bouton'-like profiles (Fig 1a). Relatively rare, they were always located in the dorsal confines of the nucleus. When the immunoprecipitate was lightly distributed, it was possible to see that such profiles were not dendrites, as they contained no trace of rough endoplasmic reticulum or ribosomes, but numerous small clear vesicles, ~50 nm in diameter (Figs 1, 2). More infrequently, they also contained dense-cored vesicles (Fig. 1a, b). Several of these immunoreactive terminals established synaptic contacts (characterized by pre- and postsynaptic densities, enlarged synaptic clefts and associated clusters of vesicles) with dendrites (Fig. 1b, c) and somata (Fig. 2) that were also immunopositive.

These synapses resemble oxytocin-immunoreactive synapses found in extra-hypothalamic areas¹¹. All such terminals are filled with small clear vesicles and contain only a few small dense-cored vesicles (100–120 nm in diameter). In this respect, they differ from 'classical' neurosecretory terminals, as in the neurohypophysis, which show no synaptic specializations and are filled with large dense-cored secretory vesicles (180–200 nm). That our polyclonal antibody to oxytocin recognized a molecule similar to, yet different from, oxytocin remains a possibility. Proof of specificity is a problem in all immunocytochemical studies, but our observations do provide strong evidence that we have detected terminals containing true oxytocin and not some unknown immunoreactive peptide. First, the same type of terminal in the SON stained both for oxytocin and neurophysin I, its carrier protein. Second, as the oxytocin antiserum was used preabsorbed against Arg-vasopressin and identical staining

Fig. 1 Electron micrographs of oxytocin-immunoreactive terminals contacting dendrites in the rat SON. The tissue had been treated with uranyl acetate *en bloc*, but the sections had not been contrasted further, either with uranyl or lead. All highly electron-dense deposits represent peroxidase reaction product resulting from the immunocytochemical staining. No detergents were used during the immunocytochemical reaction. Immunoprecipitate is seen in varying amounts (due to variability in penetration of the immunoreagents or to different concentrations of the immunoreactive peptides) in the cytoplasm of the terminals (asterisks) and dendrites (d). The immunopositive endings contain numerous small round vesicles and, at times, several larger dense-cored vesicles, which unlike the former, are covered with immunoprecipitate (arrows, *a*, *b*). In *b* and *c*, the endings are seen to form synapses (between arrowheads) onto immunopositive dendrites (d). Scale bar, 0.5 μ m.

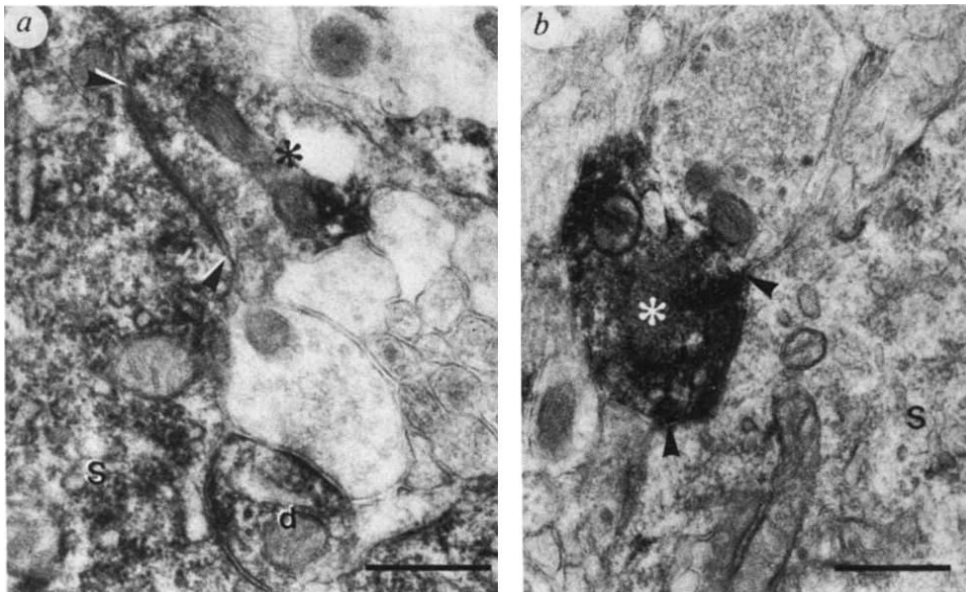
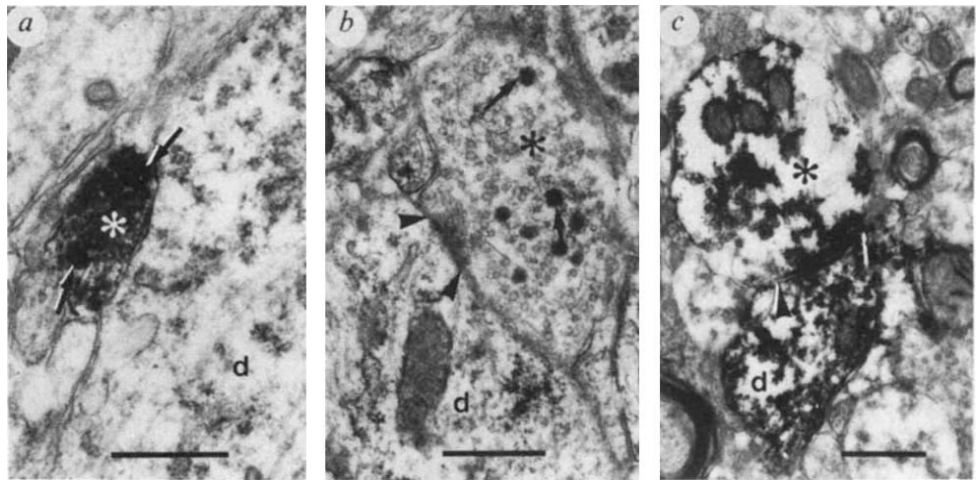


Fig. 2 Oxytocin-immunoreactive synapses onto oxytocinergic somata in the rat SON. Such synapses (asterisks) are here covered by dense deposits of immunoprecipitate, but the synaptic specializations are still visible (between arrowheads). This allows them to be distinguished from small immunopositive dendritic profiles (d, *a*). In *a*, the soma (S) is also heavily labelled whereas in *b* it contains only sparse deposits of immunoprecipitate. In all electron micrographs, note that the surrounding neuropil is completely devoid of peroxidase reaction product. Scale bar, 0.5 μ m.

was observed in genetically vasopressin-deficient Brattleboro rats, the staining was not due to vasopressin. Finally, our sera did not crossreact with a variety of peptides (unrelated to the neurohypophysial peptides) of different amino acid composition, on sections or on our immunocytochemical models.

The striking feature of our observations is that oxytocin-immunoreactive neurones establish synaptic connections between each other which could be of functional significance to the oxytocin system as a whole. During lactation, the whole population of oxytocin neurones in the four magnocellular nuclei acts synchronously to induce a pulsatile release of hormone before each reflex milk ejection¹². Interconnections between the neurones may permit this synchronization, either between oxytocin cells within the same nucleus (oxytocin cells give off axon collaterals in the dorsal area of the SON¹³) or between cells of the different magnocellular nuclei in the hypothalamus¹⁴. That the dense-cored vesicles contained in the terminals were smaller than those seen in magnocellular neurones argues against auto-innervation of the supraoptic somata and dendrites.

The essential question, then, is whether oxytocin is actually released from these synapses. The small clear vesicles that fill these terminals could store and release a neurotransmitter other than oxytocin. However, there is evidence that oxytocin is released, in a calcium-dependent manner, from isolated mag-

nocellular nuclei *in vitro*¹. There is also evidence that this peptide has an excitatory effect on the activity of the oxytocin neurones themselves; *in vitro*, exogenous oxytocin enhances the local release of the peptide¹; *in vivo*, it increases the electrical activity of the neurones^{4,6} and facilitates the suckling-induced release of oxytocin at milk ejection^{4,5}. The oxytocin-immunoreactive synapses described here impinged on oxytocin cells and thus could be the anatomical basis for this facilitatory postsynaptic action. The oxytocin system would thus constitute a striking example of a peptidergic system that can exert a direct positive feedback control on its activity through its own secretion.

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1. Moos, F. *et al.* *J. Endocr.* **102**, 63-72 (1984).
2. Jones, P. M., Robinson, I. C. A. F. & Harris, M. C. *Neuroendocrinology* **37**, 454-458 (1983).
3. Robinson, I. C. A. F. & Jones, P. M. *Neuroendocrinology* **34**, 60-64 (1982).
4. Freund-Mercier, M. J. & Richard, P. *Neurosci. Lett.* **23**, 193-198 (1981).
5. Freund-Mercier, M. J. & Richard, P. *J. Physiol., Lond.* **352**, 447-466 (1984).
6. Moss, R. L., Dyball, R. E. J. & Cross, B. A. *Expl. Neurol.* **34**, 95-102 (1972).

7. Sternberger, L. A. *Immunocytochemistry* (Prentice-Hall, Englewood Cliffs, New Jersey, 1974).
8. Theodosis, D. T. & Poulain, D. A. *Cell Tissue Res.* **235**, 217-219 (1984).
9. Bulet, A., Tonon, M. C., Tankosic, P., Coy, D. & Vaudry, H. *Neuroendocrinology* **37**, 64-72 (1983).
10. Theodosis, D. T. *Brain Res.* **233**, 3-16 (1982).
11. Buijs, R. M. & Swaab, D. F. *Cell Tissue Res.* **204**, 355-365 (1979).
12. Poulain, D. A. & Wakerley, J. *Neuroscience* **7**, 773-808 (1982).
13. Mason, W. T., Ho, Y. W. & Hatton, G. I. *Neuroscience* **11**, 169-182 (1984).
14. Silverman, A. J., Hoffman, D. L. & Zimmerman, E. A. *Brain Res. Bull.* **6**, 47-61 (1981).

Interferon amplifies complement activation by Burkitt's lymphoma cells

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Interferon was originally described as an antiviral agent produced shortly after onset of infection with most viruses¹. However, in addition to inducing an antiviral state, interferon inhibits cell division², increases the expression of cell-surface antigens³, boosts the cytotoxic activity of natural killer (NK) cells^{4,5} and modulates several immune functions of lymphocytes and macrophages⁶⁻⁹. Moreover, a special class of interferon (immune interferon or IFN- γ) is produced by T cells following stimulation with antigen or interaction with mitogens^{10,11}. The different methods by which interferon is induced and its multiple effects suggest that it may be part of a first-line defence system controlling the spread of virus infections and the proliferation of modified 'self' cells that have been affected by virus infection or neoplastic transformation¹². The ability of certain human lymphoma cells to activate the alternative pathway of complement is well established¹³⁻¹⁵. Here we show that monoclonal antibody-purified interferon can amplify the ability of certain tumour cells to activate complement via the alternative pathway. This demonstration may reflect an additional, as yet unknown, role of interferon in inducing non-specific anti-tumour immunity.

The Epstein-Barr virus (EBV)-carrying Burkitt's lymphoma (BL) line Raji (ref. 16), the EBV-free BL line BJAB (ref. 17) and the EBV-converted line BJAB/B958 (ref. 18) were cultured in the presence of affinity-purified human α -interferon (IFN- α). Interferon-treated cells and untreated controls were examined for their ability to activate the third component of complement (C3) using a two-dimensional crossed immunoelectrophoresis assay. Human hypogammaglobulinaemic serum (as a source of C3) was used throughout and C3 activation was carried out in the presence of EGTA and Mg^{2+} which blocks classical pathway activation but supports alternative pathway activation, or in the presence of EDTA which blocks both activation pathways.

Figure 1 shows that Raji cells cultured in 2,500 U ml⁻¹ IFN- α activate C3 to a greater extent than untreated control cells (38.9% as opposed to 15.7% C3 conversion, respectively). No C3 conversion was observed in the presence of EDTA. The kinetics of increased alternative pathway activation by interferon-treated Raji cells is shown in Fig. 2. Increased C3 conversion was first detected on day 2 of culture. Removal of interferon on day 4 resulted in a decreased activation on day 5 and a complete reversal of the effect on day 6. Cultures with and without interferon proliferated equally well, as Raji cells are resistant to the cell growth inhibitory effect of interferon².

The levels of conversion of C3 by several interferon-treated lines are summarized in Table 1. Increased C3 conversion was

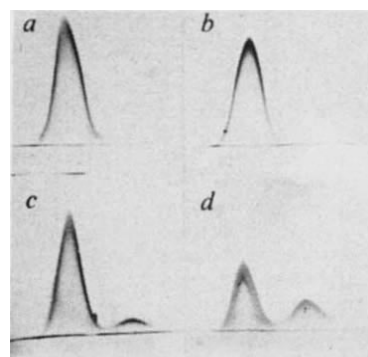


Fig. 1 Alternative pathway C3 activation by interferon-treated Raji cells. Cells were cultured for 3 days in RPMI 1640 medium containing 10% fetal bovine serum (Gibco) with or without 2,500 U ml⁻¹ IFN- α , which was affinity-purified on a column of monoclonal anti-IFN- α antibodies as described elsewhere²³. To determine alternative pathway activation, a human hypogammaglobulinaemic serum was made to 0.01 M EGTA and 0.001 M $MgCl_2$ (serum/EGTA- Mg^{2+}) or to 0.01 M EDTA (serum/EDTA). Cells were washed twice, then 10^6 cells were incubated with 25 μ l of serum/EGTA- Mg^{2+} or serum/EDTA at 37 °C for 30 min. The reaction was stopped by adding 25 μ l of 0.02 M EDTA and the cells were centrifuged. The supernatants were collected and assayed for C3 conversion in a two-dimensional crossed immunoelectrophoresis assay as described elsewhere²⁴. The first dimension was run in 2% agar (Difco) using Veronal buffer containing 0.01 M EDTA (pH 8.6) at 5 mA cm⁻¹ for ~3 h. The second dimension was run against sheep anti-human C3 antiserum at 5 mA cm⁻¹ overnight. The plates were dried, stained in 0.1% Coomassie brilliant blue and destained in methanol/acetone (1:1). The main peak represents native C3 and the smaller, faster-migrating peak represents the C3b conversion product. Per cent conversion was determined by measuring the relative area under the C3b peak. *a*, Raji cells with serum/EDTA (0% conversion); *b*, interferon-treated Raji cells with serum/EDTA (0% conversion); *c*, Raji cells with serum/EGTA- Mg^{2+} (15.7% conversion); *d*, interferon-treated Raji cells with serum/EGTA- Mg^{2+} (38.9% conversion).

detected when Raji cells were treated with either 1,250 or 2,500 U ml⁻¹ of IFN- α . The EBV-free BJAB line and the EBV-converted BJAB/B958 subline also showed increased alternative pathway activation following culture with interferon. No C3 activation by human peripheral blood lymphocytes (PBL) was observed.

Raji cells which have activated complement become coated with C3b on their membranes, which can be detected on the cell surface by fluorescein isothiocyanate (FITC)-conjugated anti-C3 antibodies¹⁹. We therefore examined the amount of C3b fixed on interferon-treated Raji cells following alternative pathway activation. Cells treated with IFN- α showed an increased fluorescence intensity compared with untreated control cells (Fig. 3), indicating that interferon also augments the amount of membrane-bound C3b on the C3 activating cell. EDTA completely blocked the membrane deposition of C3b.

It is important to exclude the possibility that the increased amount of membrane-bound C3b on the C3 activating cells is not due simply to an increase in the level of C3b receptor (CR1) expression by the interferon-treated cells. To test for a quantitative increase in CR1, both Raji cells and interferon-treated Raji cells (IFN-Raji) were incubated in fluid-phase C3b (generated by zymosan treatment of serum), then the cells were washed and stained with FITC-conjugated anti-human C3. This method allows the detection of CR1 as C3b has lost its metastable binding site and can bind only to the receptors. The fluorescence-activated cell sorting (FACS) profiles for Raji and IFN-Raji were essentially identical (data not shown), proving that interferon does not affect CR1 expression. The mean fluorescence intensity for Raji cells was 35.72 ± 0.40 and 79.6% of cells were positive; the corresponding values for interferon-treated Raji cells were 36.07 ± 0.40 and 80.1%.

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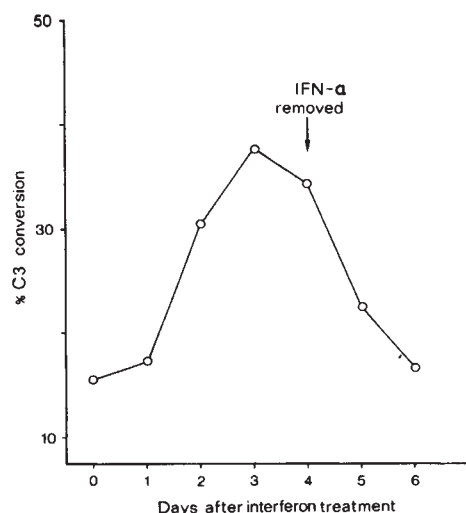


Fig. 2 Kinetics of acquisition and reversibility of increased alternative pathway C3 activation by interferon-treated Raji cells. Cells were cultured in medium containing $2,500 \text{ U ml}^{-1}$ IFN- α . IFN- α was removed from the culture at day 4 and the cells were cultured for an additional 2 days. The cells were assayed daily for C3 conversion as described in Fig. 1 legend.

To further exclude the possibility that C3b was bound only to cell membrane-associated CR1 receptors in the above experiments in which C3 was detected on the membrane after activation, both the Raji and IFN-Raji cells were checked by immune adherence using CR1-bearing erythrocytes. By this assay, 85% of Raji cells were shown to be immune adherence-positive compared with 89% after interferon treatment. However, this assay detects only qualitative and not quantitative changes in the amount of C3b bound and as Raji cells activate complement, albeit at a lower level, most of the cells are immune adherence-positive after incubation in serum under activating conditions even before interferon treatment.

Thus, it seems that interferon can augment the ability of certain tumour cells to activate and fix autologous C3. Previous studies have established that alternative pathway activation by EBV-transformed human B lymphoma cells is antibody-independent^{13,20}. This type of activation may be part of a nonspecific mechanism by which altered self cells are being controlled and eliminated. Fixation of C3b by cells activating the alternative pathway can lead to subsequent activation of the terminal pathway and hence elimination by the reticuloendothelial system. Augmentation of alternative pathway activation is also known

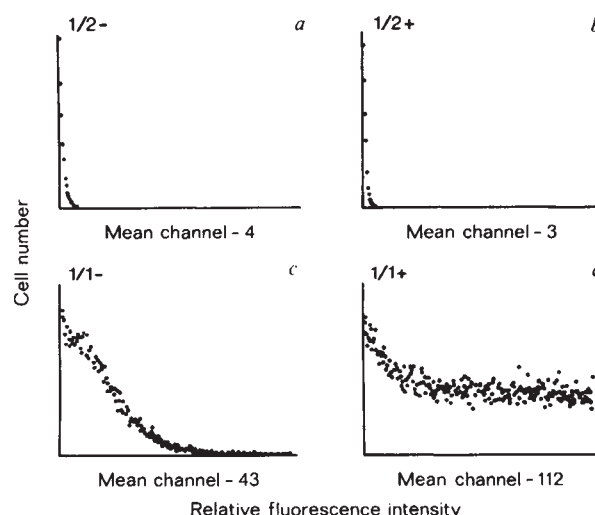


Fig. 3 Cytofluorimetric analysis of C3b fixation on Raji cells. *a*, Raji with serum/EDTA; *b*, interferon-treated Raji with serum/EDTA; *c*, Raji with serum/EGTA- Mg^{2+} ; *d*, interferon-treated Raji with serum/EGTA- Mg^{2+} . Cells cultured for 3 days with or without $2,500 \text{ U ml}^{-1}$ IFN- α were incubated with either serum/EGTA- Mg^{2+} or serum/EDTA (30 min at 37°C) followed by FITC-conjugated goat anti-human C3($\beta 1\text{c}/\beta 1\text{a}$) antibodies (Hyland Laboratories, Los Angeles) at 4°C for 30 min. After each treatment the cells were washed twice and finally resuspended and analysed in a flow cytometer (FACS II; Becton-Dickinson) for fluorescence intensity profiles.

to occur following infection of cells by murine leukaemia viruses²¹ or by EBV²⁰. As viral infections of cells result in interferon production, it is interesting to speculate that the increased C3 conversion is actually mediated by interferon. In this regard, Table 1 shows that although non-interferon-treated EBV-transformed BJAB/B958 cells produce greater activation than EBV-negative BJAB, as has been described previously²⁰, the difference between the lines is not apparent after interferon treatment. Another question of interest is the relationship between the phenomenon described here and the ability of interferon to modulate NK activity and sensitivity²², and to what extent complement and complement activation may participate in the NK lytic event. These questions merit further study.

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Table 1 Effect of IFN- α on alternative pathway C3 conversion by several BL cell lines and PBL

Cells	IFN (U ml^{-1})	% C3 conversion*
Raji	—	15.7, 16.4, 14.8
	1,250	36.0, 34.8, 37.0
	2,500	38.9, 38.4, 33.2
BJAB	—	0, 3.4
	1,250	22.7, 18.3
	2,500	26.1, 17.6
BJAB/B958	—	9.4, 13.5
	1,250	19.0, 23.9
	2,500	23.5, 27.7
PBL	—	0, 0
	1,250	0, 0
	2,500	0, 0

Cells were cultured with or without IFN- α for 3 days, then tested for C3 conversion as described in Fig. 1 legend. PBL, peripheral blood lymphocytes obtained from heparinized blood and separated on a Ficoll-Hypaque gradient.

* Results of individual experiments.

- Isaacs, A. & Lindenman, J. *Proc. R. Soc. B* **147**, 258-267 (1957).
- Adams, A., Strander, H. & Cantell, K. *J. gen. Virol.* **28**, 207-217 (1975).
- Lindahl, P., Gresser, I., Leary, P. & Tovrey, M. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1284-1287 (1976).
- Gidlund, N., Orn, A., Wiggzell, H., Senik, A. & Gresser, I. *Nature* **273**, 759-761 (1978).
- Djeu, J. Y., Heinbaugh, J. A., Holden, M. T. & Herberman, R. B. *J. Immun.* **22**, 175-181 (1979).
- Gisler, R. H., Lindahl, P. & Gresser, I. *J. Immun.* **113**, 438-444 (1974).
- Leanderson, T., Hillorn, V., Holmberg, D., Larsson, E. L. & Lundgren, E. *J. Immun.* **129**, 490-494 (1982).
- Donahoe, K. M. & Hung, K. Y. *Infect. Immun.* **13**, 1250-1257 (1976).
- Szigeti, R. *et al. Nature* **288**, 594-595 (1980).
- Epstein, L. B., Stevens, D. A. & Merigan, T. C. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2632-2636 (1972).
- Epstein, L. B., Kerth, W. H. & Herzenberg, L. A. *Cell. Immun.* **12**, 407-421 (1974).
- Sonnenfeld, G. in *Lymphokine Rep.* Vol. 1 (ed. Pick, E.) 113-131 (1980).
- Budzko, D. B., Lachmann, P. J. & McConnell, I. *Cell. Immun.* **22**, 98 (1976).
- Theofilopoulos, A. N. & Perrin, L. H. *J. exp. Med.* **143**, 271 (1976).
- Schreiber, R. D., Pangburn, M. K., Medicus, R. G. & Müller-Eberhard, H. J. *Clin. Immun. Immunopath.* **15**, 384 (1980).
- Epstein, M. A. *et al. J. natn. Cancer Inst.* **37**, 547-559 (1966).
- Menezes, J., Leibold, W., Klein, G. & Clements, G. *Biomedicine* **22**, 267-284 (1975).
- Fresen, K. O. & Zur Hausen, H. *Int. J. Cancer* **17**, 161-166 (1976).
- Yefenof, E., Kledin, G. & Kuernung, K. *Cell. Immun.* **25**, 225-233 (1977).
- McConnell, I., Klein, G., Lint, T. F. & Lachmann, P. J. *Eur. J. Immun.* **8**, 453-458 (1978).
- Okadao, H. & Baba, T. *Nature* **248**, 521-522 (1974).
- Trinchieri, G. & Santoli, D. J. *exp. Med.* **147**, 1314-1333 (1978).
- Secher, D. S. & Burke, D. C. *Nature* **446-450** (1980).
- Lachmann, P. J. & Hobart, M. J. in *Handbook for Experimental Immunology* 3rd edn (ed. Weir, D. M.) Ch. 5A (Blackwell Scientific, Oxford, 1978).

Phytohaemagglutinin activation of T cells through the sheep red blood cell receptor

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Expression of receptors for sheep red blood cells and the ability to proliferate in response to phytohaemagglutinin (PHA) are the traditional properties of human T cells^{1,2}, but the function of the sheep red cell receptor (the T11 antigen) is controversial^{3,4} and the mechanism of PHA-induced mitogenesis unclear. Mitogenesis involves a complex series of cell-mediated and factor-dependent interactions, but a rise in intracellular free calcium concentration, $[Ca^{2+}]_i$, seems to be an important primary event in T-cell activation⁵⁻⁷. We have now investigated the effects of three monoclonal antibodies, previously shown to inhibit mitogen-induced proliferation^{3,8,9}, on T-cell $[Ca^{2+}]_i$. We find that anti-LFA-2 and OKT11, which react with the sheep red cell receptor^{8,10}, have no effect on $[Ca^{2+}]_i$, nor do they inhibit the rise in $[Ca^{2+}]_i$ induced by concanavalin A (Con A) or the mitogenic anti-T3 monoclonal antibody UCHT1 (ref. 11). They do, however, block PHA-induced Ca^{2+} mobilization. Anti-LFA-1, which reacts with the lymphocyte function-associated antigen^{8,12}, has no effect on intracellular Ca^{2+} . These studies suggest that the sheep red blood cell receptor is an activation pathway for T cells and that the effects of PHA are mediated through this pathway.

We have shown previously that Con A and PHA produce a rise in $[Ca^{2+}]_i$ within 2-3 min of addition¹³. Similar effects can be induced in T cells or T-cell lines by monoclonal antibodies to the T3-antigen receptor complex^{13,14}, and there is considerable evidence that this complex is an important pathway for T-cell activation (for a review see ref. 15). Induction of a rise in $[Ca^{2+}]_i$ by Con A, PHA and anti-T3 antibodies requires extracellular Ca^{2+} and can be reversibly blocked by the addition of EGTA to the extracellular fluid (data not shown). Although this suggests that there is an influx of Ca^{2+} across the cell membrane, a Ca^{2+} -dependent mechanism leading to the release of Ca^{2+} from intracellular stores cannot be excluded and the term Ca^{2+} mobilization is thus used here.

Neither anti-LFA-1 nor anti-LFA-2 or OKT11 stimulated an increase in $[Ca^{2+}]_i$ during the 10-min observation period, when added at various concentrations determined by indirect immunofluorescence to be fully saturating. We have demonstrated previously that one T-cell mitogen, the monoclonal antibody WT-31, requires crosslinking to stimulate Ca^{2+} mobilization in cloned T cells¹⁴, but investigation of the effect of crosslinking with anti-immunoglobulin after binding of each of these antibodies revealed no effect on $[Ca^{2+}]_i$ (data not shown). Fully saturating or higher concentrations of the control antibodies 2A1 (anti-HLA-A, B, C)¹⁶ and anti-Hle-1 (anti-common leukocyte antigen)¹⁷ also failed to produce a rise in $[Ca^{2+}]_i$, either when added alone or when subsequently crosslinked.

In all these experiments, binding of any of the above antibodies (with or without crosslinking) did not prevent the cells responding normally to Con A or UCHT1 when either mitogen was added subsequently. The kinetics and magnitude of these responses were identical with those of T cells treated with mitogen alone (Fig. 1).

The binding of anti-LFA-1 or the control antibodies had no effect on the ability of PHA to elicit Ca^{2+} mobilization in T

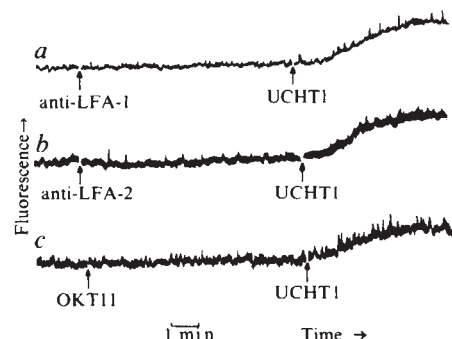


Fig. 1 Response of T cells to anti-LFA-1 (a), anti-LFA-2 (b), OKT11 (c) and UCHT1. T cells were prepared from peripheral blood mononuclear cells as described previously¹⁴. Cells (10^7) were incubated at 37 °C with 15 μ M Quin 2 AM (Amersham) in RPMI 1640 (Gibco) for 60 min with a fivefold dilution after 20 min. They were washed twice and re-suspended in a buffered saline solution¹⁴ before fluorescence measurements. Quin 2 fluorescence was measured on a standard fluorimeter (Locarte, London) using an excitation wavelength of 339 nm and measurement of emission at 492 nm. Cuvettes were kept at 37 °C and cell suspensions were continuously stirred during fluorescence measurements. OKT11 was obtained from Ortho Diagnostic Systems. All antibodies were used at greater than saturating concentrations, as shown by indirect immunofluorescence staining and flow cytometric analysis on a FACS-IV (Becton Dickinson). Affinity-purified rabbit anti-mouse IgG (heavy and light chains) was obtained from Zymed Laboratories, San Francisco. Concanavalin A (Con A, Miles-Yeda) was used at a concentration of 20 μ g ml⁻¹, which was found to be optimal for proliferation and the ability to stimulate maximum Ca^{2+} mobilization. Neither anti-LFA-1 (trace a), anti-LFA-2 (trace b) nor OKT11 (trace c) induce Ca^{2+} mobilization either when added alone or when subsequently crosslinked (data not shown). UCHT1 (or Con A, not shown) could still induce Ca^{2+} mobilization normally after addition of any of these agents.

cells, nor did crosslinking of these antibodies on the cell surface have any effect on subsequent stimulation with PHA.

Although anti-LFA-2 and OKT11 binding, with or without crosslinking, had no effect on Con A or UCHT1 stimulation of T cells (Fig. 1), the ability of PHA to stimulate Ca^{2+} mobilization was markedly reduced by the prior binding of these antibodies (Table 1, Fig. 2). This phenomenon was dose-dependent. A 1:800 dilution had no inhibitory effect on PHA stimulation; successively increasing concentrations of anti-LFA-2 had greater effect and a 1:100 dilution completely abrogated the PHA response. This inhibitory effect was specific for PHA, as $[Ca^{2+}]_i$ increased normally in response to subsequent UCHT1 or Con A. Responses to concentrations of Con A or UCHT1 which induced suboptimal Ca^{2+} mobilization were also unaffected by prior treatment with anti-T11 antibody. The T-cell lines HUT 78 (ref. 18) and HPB-ALL (ref. 19) also showed anti-LFA-2 induced blockade of PHA-induced Ca^{2+} mobilization (data not shown).

Our finding that anti-LFA-1 has no effect on the ability of Con A, PHA or UCHT1 to elicit an increase in $[Ca^{2+}]_i$ in either peripheral blood T-cells or the T-cell lines HUT78 and HPB-ALL indicates that the antibody prevents proliferation by a mechanism not involving early Ca^{2+} mobilization. It has been shown that anti-LFA-1 and anti-LFA-2 inhibit interaction between cytotoxic T lymphocytes and target cells⁸, and similar cell-contact inhibition between T cells and accessory cells may account for the inhibition observed with anti-LFA-1.

We have shown previously that T cells from a proportion of normal individuals do not undergo a proliferative response to IgG1 anti-T3 antibodies and yet exhibit a normal $[Ca^{2+}]_i$ rise to UCHT1¹⁴. The observation that anti-LFA-1 antibody does not block Ca^{2+} mobilization, but inhibits proliferation⁸, confirms the finding that a rise in $[Ca^{2+}]_i$ alone is not sufficient to induce proliferation; secondary signals probably provided by T-cell/accessory cell and T-cell/T-cell interactions are also required. Note that the conditions used to measure $[Ca^{2+}]_i$ are such that cell interaction is minimal¹³. Furthermore, the aug-

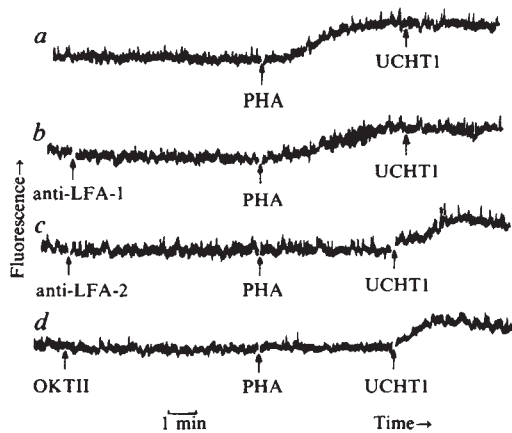


Fig. 2 Response to PHA (a) after addition of anti-LFA-1 (b), anti-LFA-2 (c) and OKT11 (d). Quin 2-loaded T cells were treated with $2 \mu\text{g ml}^{-1}$ of purified phytohaemagglutinin (PHA, Wellcome) which was the concentration found to stimulate maximal proliferation and Ca^{2+} mobilization. Trace a shows a normal PHA response. Subsequent UCHT1 addition had no further effect. Trace b shows that pretreatment of cells with anti-LFA-1 had no effect on the subsequent PHA response. Traces c and d show that pretreatment of T cells with anti-LFA-2 or OKT11 abrogated Ca^{2+} mobilization to PHA but not the ability of UCHT1 to stimulate a subsequent response. Con A produced similar results if substituted for UCHT1 in these experiments (data not shown).

mentation of proliferation induced by phorbol myristate acetate (PMA) and interleukin-2 do not involve early Ca^{2+} mobilization^{7,14}.

Palacios and Martinez-Maza showed that OKT11A, an antibody to the sheep red cell receptor, blocked proliferation induced by antigen, Con A, OKT3 and the calcium ionophore A23187, but not by PMA³. As PMA apparently induces proliferation through a Ca^{2+} -independent pathway⁷, they postulated that the sheep red cell receptor is a 'negative signal receptor', and that antibodies to this molecule prevent Ca^{2+} mobilization on subsequent stimulation. The present study shows clearly, however, that the anti-T11 antibodies (anti-LFA-2 and OKT11) do not prevent the cells from mobilizing Ca^{2+} normally on addition of Con A or the anti-T3 antibody UCHT1 (Fig. 2, Table 1). Inhibition of these proliferative responses may again be at the accessory cell level. The anti-T11 antibodies do, however, markedly suppress the Ca^{2+} response to subsequent PHA addition (Fig. 2, Table 1). This binding of antibody to the T11 receptor followed by PHA addition does not prevent either UCHT1 or Con A from mobilizing Ca^{2+} (Fig. 2), indicating that the T11 antigen is not a general negative signal receptor. A more likely explanation is that PHA stimulates T cells via the T11 antigen, resulting in Ca^{2+} mobilization and ultimately proliferation, and that PHA interaction with this molecule is blocked by some anti-T11 antibodies.

These findings agree with the recent work of Meuer *et al.*⁴, who propose that the T11 antigen represents an alternative pathway of T-cell activation. These workers have identified three

distinct epitopes on the sheep red cell receptor which they have named T11₁, T11₂ and T11₃. T11₁ is the sheep red cell binding site and both the T11₁ and T11₂ epitopes are present on all T cells. T11₃, however, is expressed only after T-cell activation. Proliferation requires binding of both T11₂ and T11₃ epitopes by monoclonal antibodies. We suggest that PHA binds to the T11 molecule, including the T11₂ epitope, and in doing so, produces a conformational change necessary to expose the T11₃ site. PHA then binds to this epitope, resulting in Ca^{2+} mobilization and ultimately proliferation. This explains why the antibodies to the sheep red cell receptor binding site block PHA responses and do not themselves cause the Ca^{2+} mobilization observed with antibodies to the T3 complex.

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- Greaves, M. F. & Janossy, G. *Transplant Rev.* **11**, 87-130 (1973).
- Coombs, R. A., Gumer, B. W., Wilson, A. B., Holm, G. & Lindgren, B. *Int. Archs Allergy appl. Immun.* **39**, 658-663 (1970).
- Palacios, R. & Martinez-Maza, O. *J. Immun.* **129**, 2479-2485 (1982).
- Meuer, S. C. *et al. Cell* **36**, 897-906 (1984).
- Lichtman, A. H., Segal, G. B. & Lichtman, M. A. *Blood* **61**, 413-422 (1983).
- Metcalfe, J. C., Pozzan, T., Smith, G. A. & Hesketh, T. R. *Biochem. Soc. Symp.* **45**, 1-26 (1980).
- Tsien, R. Y., Pozzan, T. & Rink, T. J. *Nature* **295**, 68-71 (1982).
- Krensky, A. M. *et al. J. Immun.* **131**, 611-616 (1983).
- Martin, P. J. *J. Immun.* **131**, 180-185 (1983).
- Verbi, W. *et al. Eur. J. Immun.* **12**, 81-86 (1981).
- Beverly, P. C. L. & Callard, R. E. *Eur. J. Immun.* **11**, 329-344 (1981).
- Sanchez-Madrid, F. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 7489-7493 (1982).
- O'Flynn, K., Linch, D. C. & Tatham, P. E. R. *Biochem. J.* **219**, 661-666 (1984).
- O'Flynn, K. *et al. Eur. J. Immun.* (in the press).
- Beverly, P. C. L. *Nature* **304**, 398-399 (1983).
- Beverly, P. C. L. *Proc. R. Soc. Edinb.* **B81**, 221-232 (1982).
- Beverly, P. C. L., Linch, D. C. & Delia, D. *Nature* **287**, 332 (1980).
- Gazdar, A. F. *et al. Blood* **55**, 409-417 (1980).
- Minowada, J., Sagawa, K., Trowbridge, I. S., Kung, P. D. & Goldstein, G. in *Malignant Melanomas* (eds Rosenberg, S. A. & Kaplan, H. S.) 53-74 (Academic, New York, 1982).

Critical test of a sister chromatid exchange model for the immunoglobulin heavy-chain class switch

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Table 1 Rise in $[\text{Ca}^{2+}]_i$ on addition of mitogens, expressed as a percentage of resting $[\text{Ca}^{2+}]_i$

Prior incubation	Mitogen	Rise in $[\text{Ca}^{2+}]_i$	n
—	PHA	$70.5 \pm 26\%$	14
—	UCHT1	$64.9 \pm 26\%$	7
Anti-LFA-2	PHA	$8.5 \pm 8.3\%$	13
Anti-LFA-2	PHA + UCHT1	$66.2 \pm 25\%$	14

$[\text{Ca}^{2+}]_i$ was determined using the Triton X-100/DTPA/MnCl₂ calibration technique described previously¹³. Resting $[\text{Ca}^{2+}]_i$ was determined to be $115 \text{ nM} \pm 45 \text{ (s.d.)}$ ($n = 13$). Post-stimulation $[\text{Ca}^{2+}]_i$ was measured 4 min after addition of mitogen.

B lymphocytes may switch from producing an immunoglobulin heavy chain of the μ class to that of the γ , ϵ or α class¹⁻⁴. To maintain the specificity, the new heavy chain must keep the original variable (V) region; this is achieved by deleting DNA sequences so that the V (consisting of joined V_H , diversity (D_H) and joining (J_H) gene segments) and C (constant) gene segments coding for the new heavy chain are brought into close proximity (reviewed in ref. 5; we do not consider here the μ - δ situation). There are, in principle, three types of chromosomal rearrangements that yield a deletion: (1) rearrangement within a chromatid; (2) unequal sister chromatid exchange (as suggested by Obata *et al.*⁶); and (3) unequal recombination between chromosomal homologues. We have analysed the arrangement of C_μ DNA in clones of the pre-B-cell line 18-81 that switches *in vitro* from μ to $\gamma 2b$ (ref.

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7). The clones examined produce either μ , $\gamma 2b$ or no immunoglobulin chain. We report here that all the $\gamma 2b$ clones had lost at least one copy of C_μ and no clones contained three copies of C_μ . These findings formally exclude both unequal sister chromatid exchange and recombination between homologues as mechanisms for creating a gene encoding the $\gamma 2b$ chain.

Figure 1 shows an unequal sister chromatid exchange mechanism and the predicted genotypes of the progeny cells which would be produced in cell line 18-81. The two different V alleles of 18-81 are designated V2 and V3 because they contain J2 and J3, respectively^{8,9}. The silent allele is V2, the active V3 (refs 7, 8). Two new types of cells are generated: $\gamma 2b$ cells containing only one C_μ gene segment located on the silent homologue and in close proximity to V2, and an equivalent number of segregant cells containing three C_μ gene segments. Two of the C_μ segments are located on the productive homologue, with one connected to V3 and the other to no V; one C_μ is located on the silent homologue and is connected to V2. As the gene for μ production is unaltered, these cells should continue to produce μ chain.

Recombination between homologues as a mechanism for switching of heavy-chain class production has already been excluded at the cellular level¹⁰, and also *in vivo* by the fact that the V- and C-region allotypes of a given immunoglobulin molecule are generally both encoded by one homologue¹¹⁻¹³. In its simplest form, recombination would generate three new types of cells of equal frequency: $\gamma 2b$ cells containing one C_μ , $\gamma 2b$ cells containing two C_μ segments on the silent homologue and μ cells containing three C_μ segments. In the cell line A3, derived from 18-81 and switching from μ to $\gamma 2b$ *in vitro*, there are no $\gamma 2b$ cells containing two C_μ segments⁷, excluding recombination between homologues as the mechanism of the switch *in vitro*.

As unequal sister chromatid exchange would produce segregant cells containing three copies of C_μ , in numbers equal to the $\gamma 2b$ cells, and as there is no obvious reason why these cells should not be viable, they should be found in the population of a clone in which some cells have switched from μ to $\gamma 2b$ production. Of 103 subclones of clone A3 analysed, three produced no immunoglobulin chain, 91 produced μ and nine produced $\gamma 2b$. At least seven of the nine $\gamma 2b$ clones should have resulted from independent switching events, because on Southern blot analysis, both *Xba*I and *Bam*HI restriction fragments containing the active V3 region (restriction map given in Fig. 2) and *Sac*I restriction fragments containing part of the $C_{\gamma 2b}$ are of different sizes (*Xba*I fragments in Fig. 3a). We believe that the difference in size is caused by switch rearrangements at different sites.

As nine of the 103 subclones produced $\gamma 2b$, one would expect to find nine subclones containing three copies of C_μ if unequal sister chromatid exchange produced the switch in heavy chain class. We screened for an additional C_μ on the active (or silent)

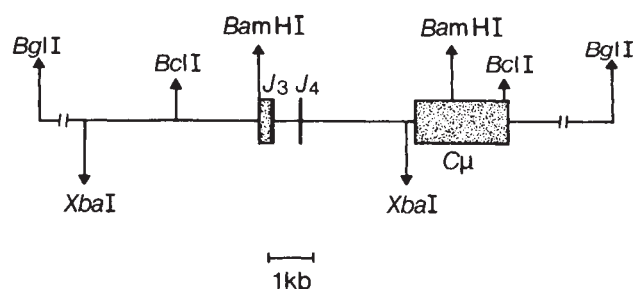


Fig. 2 Restriction map of the V3 allele in line A3 before the heavy-chain class switch. There is a 5.4-kilobase (kb) deletion in the J_H - C_μ intron including two *Xba*I sites and the *Bgl*I site. The two *Bgl*I sites indicated are more than 20 kb apart.

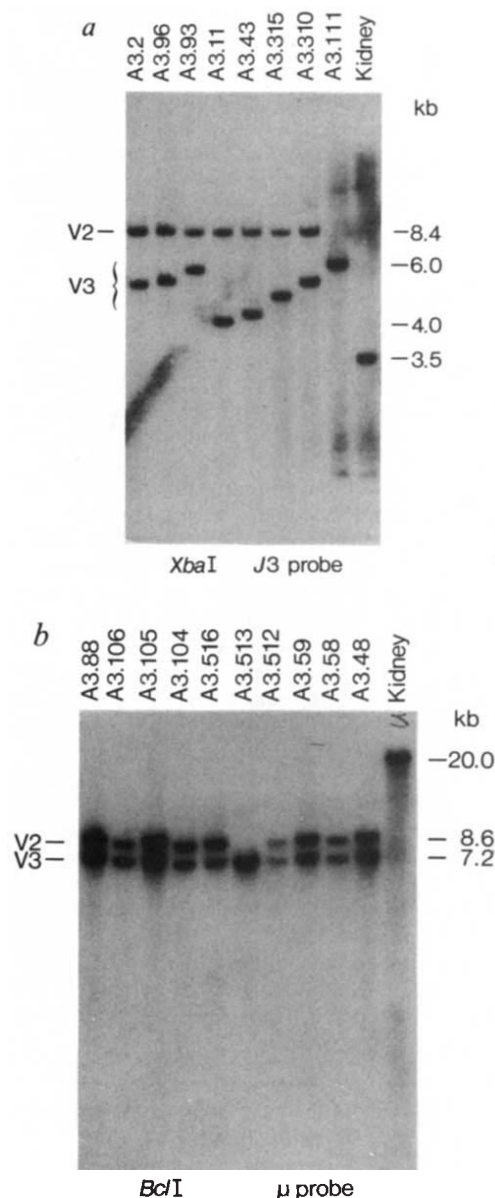


Fig. 3 Southern blot analysis of DNA of $\gamma 2b$ -producing (a) and μ -producing (b) subclones of line A3. a, *Xba*I digests of DNA of $\gamma 2b$ -producing subclones were hybridized with a J3 probe⁷. The bands representing the V3 alleles are of different lengths in different clones. The V-region segments and the sequences 5' to them are identical (not shown). Procedures were as described previously⁷. b, *Bcl*I digests of DNAs from μ -producing subclones were hybridized with a C_μ cDNA probe⁷.

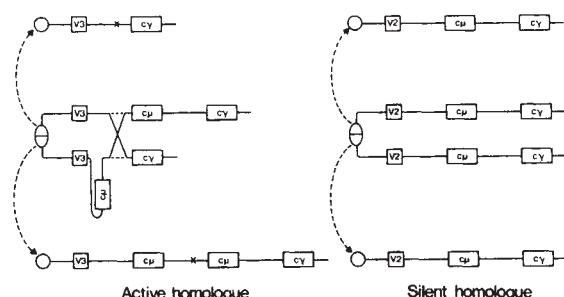


Fig. 1 Diagram of chromatid segregation in the unequal sister chromatid exchange model of the heavy-chain class switch. After recombination, the two sister chromatids of the metaphase chromosome (in the centre) are pulled apart at their centromeres. The two segregants (shown above and below the metaphase chromosome) are distributed to the daughter cells. The point of exchange is marked by X.

homologue by Southern blot analysis with the restriction enzymes *Bam*HI, *Bcl*I and *Bgl*I; these enzymes create fragments containing both *J3* and *C_μ* sequences in clone A3 (Fig. 2). Because of the recombination, the additional *C_μ* on the active homologue should be found on restriction fragments that differ in size from the *J3-C_μ* fragment in clone A3 and should not hybridize with a *J3* probe. Figure 3b shows an example of the analysis using *Bcl*I and a *C_μ* probe; in no case did we find an additional *C_μ* gene segment. The probability that we would pick nine $\gamma 2b$ subclones before finding a subclone with an extra *C_μ* segment is $(1/2)^9 = 1/512$. In a formal sense, this excludes the model of sister chromatid exchange as a means of switching of heavy-chain class.

We are aware of the possibility that, for unknown reasons, the additional *C_μ* may have been lost, but that, of course, would require an addition to the model. It is also possible that the mechanism of switching *in vivo* is different from that in a transformed line *in vitro*. Despite these reservations, however, we conclude that the deletion produced by the rearrangement of DNA is due neither to an unequal sister chromatid exchange as presented in Fig. 1 nor to unequal recombination between homologues.

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1. Pernis, B., Forni, L. & Luzatti, A. L. *Cold Spring Harb. Symp. quant. Biol.* **41**, 175-183 (1976).
2. Cooper, M. D., Kearney, J. F., Lydyard, P. M., Grossi, C. E. & Lawton, A. R. *Cold Spring Harb. Symp. quant. Biol.* **41**, 139-145 (1976).
3. Wabl, M. R., Forni, L. & Lohr, F. *Science* **199**, 1078-1080 (1978).
4. Abney, R. E., Cooper, M. D., Kearney, J. F., Lawton, A. R. & Parkhouse, R. M. E. *J. Immun.* **120**, 2041-2049 (1978).
5. Marcu, K. B. *Cell* **29**, 719-721 (1982).
6. Obata, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2437-2441 (1981).

7. Burrows, P. D., Beck-Engeser, G. B. & Wabl, M. R. *Nature* **306**, 243-246 (1983).
8. Alt, F. W., Rosenberg, N., Casanova, R. J., Thomas, E. & Baltimore, D. *Nature* **296**, 325-331 (1982).
9. Wabl, M. R., Beck-Engeser, G. B. & Burrows, P. D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 867-870 (1984).
10. Gearhart, P. J., Hurwitz, J. L. & Cebra, J. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5424-5428 (1980).
11. Landucci-Tosi, S., Mage, R. G. & Dubiski, S. J. *Immunology* **104**, 641-647 (1970).
12. Kindt, T. J., Mandy, W. J. & Todd, C. W. *Biochemistry* **9**, 2028-2032 (1970).
13. Knight, K. L. & Hanly, W. C. *Contemp. Topics molec. Immun.* **4**, 55-58 (1975).

Derivation of mouse intestinal crypts from single progenitor cells

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Adult intestinal epithelium consists of a sheet of single-cell thickness which is morphologically highly organized into tubular invaginations (crypts) and finger-like projections (villi). Proliferation of the cells is confined to the base of the crypts, from which cells migrate to the villi, where they are shed¹. The villi are formed during embryogenesis from a multilayered epithelium^{2,3}. In mice, crypts develop at about the time of birth from the epithelium between the villi, which by this stage is no longer multilayered. So far it has remained unknown how many progenitor cells contribute to each crypt, and whether they develop by the proliferation of already committed progenitors, or as a result of local inductive tissue interactions. Here, we have used mouse aggregation chimaeras as an experimental system to demonstrate immunohistochemically that the epithelium of individual crypts in small and large intestine of adult mice is always composed of cells of a single parental type. We have confirmed that this result is not an artefact of the chimaeric system by examining female mice that are mosaic for the X-linked alleles *Pgk-1^a* and *Pgk-1^b*. We conclude that the epithelium of each adult crypt is derived from a single progenitor cell.

Mouse embryo aggregation chimaeras were prepared at the MRC Laboratories, Carshalton, as described previously⁴. The mosaic cell populations in the intestinal epithelium of the chimaeras were demonstrated using H-2 antigens^{4,5} and a carbohydrate polymorphism recognized by *Dolichos biflorus* agglutinin (DBA)⁶ as markers. Table 1 shows the distribution of these markers in the strains used to construct the chimaeras. Representative areas of chimaeric small intestine stained with each marker are illustrated in Fig. 1a,b. H-2 antigens and DBA-binding sites are expressed in columnar epithelial cells, goblet

cells and Paneth cells. It is impossible, however, to ascertain at light microscope level whether these markers are also present in enteroendocrine cells, which comprise ~1% of the epithelial population. Figure 1c,d shows that corresponding mosaic patches are revealed by the H-2 and DBA markers in adjacent sections of colonic epithelium from CBA $\leftrightarrow$ C57BL/6 (H-2^k, DBA^a $\leftrightarrow$ H-2^b, DBA^a) chimaeras. We conclude that each of these markers accurately reflects the cellular genotype.

We examined histological sections of the small intestine and colon in 99 tissue samples from 34 chimaeras prepared from five different combinations of the strains listed in Table 1, including four of the congenic B10.A $\leftrightarrow$ B10-cc combination (mice were between 6 and 36 weeks old). In 55 samples from 21 chimaeras, the chimaeric components were 'balanced', that is, each genotype was estimated to contribute at least 25% of the epithelium. Crypts lying within a patch of epithelium of a single parental type would be expected to be homogeneously stained whether they arose from one or more cells; but at the border between patches of differing parental type, mixed crypts would be expected if they had been derived from more than one cell. Despite extensive patch boundaries within the tissue (Fig. 2), we found that in each chimaera, individual intestinal crypts were consistently composed of epithelial cells of only one parental type. In material estimated to comprise over 10⁵ crypts in 'balanced' chimaeric tissue samples, we have not

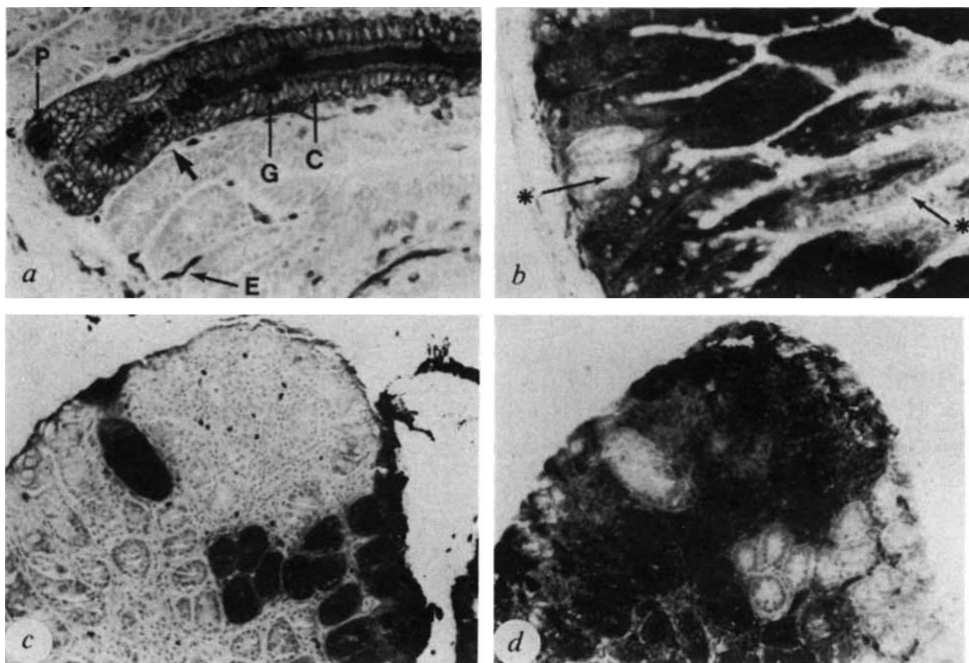
Table 1 Reactivity of inbred mouse strains with polymorphic markers

Strain	Reactivity with anti-H-2 monoclonal antibodies*	DBA binding by intestinal epithelium†
C57BL/6JLac (B6)	b	+
C57BL/10A (B10.A)	k	+
C57BL/10ScSnLac-cc (B10-cc)	b	+
CBA/CaLac (CBA)	k	+ } Distal colon
C3H/Bi (C3H)	k	+ } negative
RIII/Lac-ro (RIII)	k	—
DDK	b	—

* H-2^b, antibody FT6a9 (ref. 5); H-2^k, antibody 11.4-1 (refs 5, 9). For immunohistochemical methods, see ref. 5.

† DBA has specificity for terminal non-reducing *N*-acetylgalactosamine residues^{6,10}.

Fig. 1 DBA-binding sites and H-2 antigens as markers of chimaerism in intestinal epithelium. **a**, DBA staining. A longitudinal section of crypts (to the left of the micrograph) and villi (to the right) in chimaeric RIII $\leftrightarrow$ B6 small intestine ($\times 154$). The arrow indicates the crypt/villus junction. Methacarn-fixed, paraffin-embedded 4- μ m section. After blocking of endogenous peroxidase with 0.1% phenylhydrazine-HCl, the section was incubated with DBA-peroxidase conjugate in phosphate-buffered saline (PBS) for 1 h at room temperature, peroxidase-stained with diaminobenzidine, then counterstained with haemalum. The section was photographed on Ilford Pan-F film with a green filter (for details of method, see ref. 6). B6 epithelium (DBA⁺) appears black; RIII epithelium (DBA⁻) is unstained. In B6 epithelium, Paneth cells (P), goblet cells (G) and columnar epithelial cells (C) are all stained; the DBA-binding sites are present on cell membranes, mucin, Paneth cell granules, and in the cytoplasm, especially on the Golgi apparatus. Isolated DBA-positive cells seen in the unstained crypts are RIII endothelial cells (E). Fibroblasts and lymphocytes of neither strain bind DBA. A control section of chimaeric intestine (not shown) incubated with DBA-peroxidase conjugate in the presence of 2% *N*-acetylgalactosamine showed no staining, indicating that the DBA binding is specific. **b**, H-2 staining. Oblique section of crypts (to the left) and villi (to the right) in chimaeric CBA (H-2^k) $\leftrightarrow$ B6 (H-2^b) small intestine ($\times 130.9$); 4- μ m cryostat section, fixed in paraformaldehyde/lysine/periodate (PLP)⁵. H-2^b antigens were demonstrated using antibody FT6 α 9(monoclonal anti-H-2^b)-peroxidase conjugate; sections were stained and photographed as described for **a**. For details of method see refs 4, 5. B6 tissues, including epithelium and cells in the lamina propria, are stained. In this section two CBA crypts and CBA contributions to villus epithelium are unstained (*). Note that several villi, all cut in oblique section, are 'mixed'; they bear both stained and unstained epithelium, resulting from contributions to the villus from both H-2^b and H-2^k crypts. A reciprocal pattern (not shown) was obtained with anti-H-2^k antisera⁴. Sections of non-chimaeric B6 and CBA small intestine were present on the same slide as the chimaeric tissue in every experiment to provide a control for the specificity of H-2 staining⁷. **c, d**, Comparison of patches of CBA and B6 crypts in cross-section, visualized by DBA (**c**) and H-2 (**d**) staining. Distal colon of CBA (H-2^k, DBA⁻) $\leftrightarrow$ B6 (H-2^b, DBA⁺) chimaera; adjacent 4 μ m cryostat sections, fixed in PLP ($\times 96$). Methods as for **a**. DBA-peroxidase conjugate was used in **c**. B6 crypts are stained (the dark spots in the unstained area are mast cells, containing residual endogenous peroxidase). For **d**, 11.4-1 (anti-H-2^k)-alkaline phosphatase conjugate was used. CBA crypts are stained. Reciprocal (B6, CBA) mosaic patches are stained by each marker. Double staining of each genotype in a single section confirms that the patches are identical, but it is not shown here because of the difficulty of reproduction.



observed a single mixed crypt. (In contrast, mixed villi, resulting from the contribution of cells from more than one crypt to a single villus, are very numerous; see, for example, Fig. 1b.)

These observations suggest that the epithelium of each crypt is derived from a single progenitor cell. Before this can be accepted, however, several possibilities must be considered. It might be argued that, in aggregation chimaeras, strain differences would prevent the successful cooperation of progenitor cells of different parental type in forming a crypt. Therefore, we analysed individual colonic crypts of young adult female mice heterozygous for the X-linked alleles *Pgk-1^a* and *Pgk-1^b* and hence mosaic, due to random X-chromosome inactivation, for cells producing the variant electrophoretic isoenzymes, PGK-1A and PGK-1B (phosphoglycerate kinase, EC 2.7.2.3)⁷; the results of the electrophoretic analysis are shown in Fig. 3. Of 312 crypts dissected from the colon, 276 gave a band in the gel: 120 exhibited PGK-1A and 156 PGK-1B. As in the aggregation chimaeras, no mixed crypts were seen. Thus, our observations are unlikely to result from strain differences in the chimaeric system.

The process of crypt formation can be considered as a sampling of a number of progenitor cells from the neonatal intestinal epithelium. If the patches were very large in neonatal intestine at the time of crypt formation, samples which lay across a boundary and gave rise to mixed crypts might be so rare that we would have missed them. To examine this possibility, we estimated the patch size in neonatal intestinal epithelium and calculated the proportion of mixed crypts which would be expected (see Fig. 2 legend). Our calculations indicate that in

transects of intestinal epithelium at the time of crypt formation, patch boundaries would be expected to occur, on average, once in every 35 cells. If two cells are required to form a crypt, approximately one crypt in 35 should be 'mixed'. The chance that we would have failed to include a single mixed crypt in the 276 crypts sampled in the electrophoretic analysis of PGK mosaic mice, when mixed crypts are present in a proportion of 1 in 35, is $(34/35)^{276} = 3.3 \times 10^{-4}$. For the many thousands of crypts sampled immunohistochemically in the aggregation chimaeras, the probability that we would have missed this proportion of mixed crypts is even less: for a sample of 1,000 crypts, $< 1 \times 10^{-12}$. The failure to detect mixed crypts cannot, therefore, be explained by a lack of sufficient patch boundaries in chimaeric or mosaic intestine.

The appearance of monoclonal crypts might be obtained even though several progenitor cells were present in the crypt, if these progenitors were used as stem cells in rotation, so that the crypt epithelium at any one time was derived from only one of them. We consider this possibility unlikely, for two reasons. First, inactive progenitor cells of opposite genotypes would have remained undetected in our material only if they expressed neither H-2 antigens nor the DBA-binding site. In sections of normal intestine there is no evidence of a stem cell population which fails to express these markers. Second, such a scheme would predict the occurrence of an occasional crypt in which the differentiated epithelial cells above the basal stem cell zone are mixed, reflecting the succession of one clonal population by another of opposite genotype. Such mixed crypts would be rare, but we have not observed any.

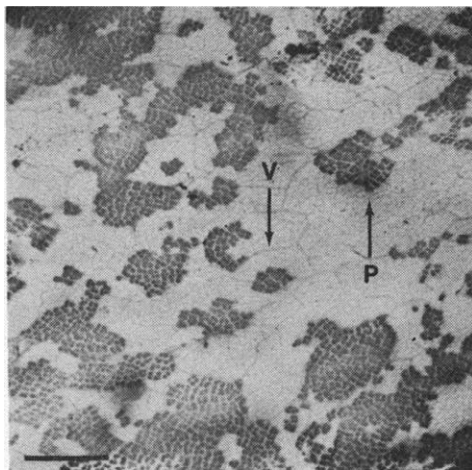


Fig. 2 Patches of crypts in chimaeric colonic epithelium. The section shows a sheet of colonic mucosa from a 6-week old RIII $\leftrightarrow$ B6 chimaera. Scale bar, 1 mm. The mucosa was dissected free of the muscles of the gut wall, pinned flat, fixed overnight in 10% buffered formol saline, then incubated for 30 min in phenylhydrazine-HCl to block endogenous peroxidase, followed by overnight incubation at 4 °C with DBA-peroxidase conjugate¹¹. Staining for peroxidase was as described in Fig. 1 legend. The sheet is viewed from the abluminal side: each darkly-stained dot is the base of a B6 crypt. RIII crypts are not stained. RIII blood vessels bind DBA-peroxidase conjugate and therefore are stained. V, RIII blood vessel; P, patch of B6 crypts. The frequency of patch boundaries in neonatal colonic epithelium and between villi in neonatal small intestinal epithelium was estimated by reconstruction: the number of patch boundaries in simulated transects of intestine from photographs of mucosal sheets such as that illustrated, was divided into the number of epithelial cells in the crypt-forming intervillus or colonic epithelium in transverse histological sections of corresponding regions of intestine from normal (non-chimaeric) neonatal mice. The measurement of numbers of patch boundaries in adult intestinal sheets is justified because the three-dimensional structure of the mucosa will prevent mixing once crypts are formed, and the number (though not the size) of patches will thus be expected to remain constant during growth of the intestine. Sixty-six simulated transects of sheets of chimaeric colon from four chimaeras (two approximately balanced 50:50, two 75:25) gave 15.1 ± 3.7 (mean $\pm$ s.d.) patch boundaries per transect, while transverse sections of mid-colon of 10 normal mice between 1 and 3 days old contained 525 ± 181 epithelial cells, giving an average of one patch boundary per 35 cells. Corresponding measurements for 24 transects of small intestine were 14.5 ± 3.7 boundaries in 445 $\pm$ 38 cells, giving one patch boundary in 31 cells. (Because of the irregular shapes and sizes of the clones which are the basic units of the mosaic pattern¹², we could not apply the analysis described by Hutchinson¹³ to determine the proportion of mixed samples expected from sampling a mosaic composed of regular units.)

Therefore, the most probable interpretation of our findings is that the epithelium of each adult crypt in chimaeras and in normal mice is descended from a single progenitor cell. Because we have not proved that enteroendocrine cells express the chimaeric markers, we cannot be certain that they share the same lineage, but indirect evidence for this exists⁸. The single progenitor may, of course, give rise to several stem cells responsible for cell renewal in the fully formed crypt.

Our results do not exclude the possibility that each crypt originally receives contributions from several progenitors at the time of crypt formation, but that the progeny of one always displaces the others in early postnatal life. Information on this point will be obtained from examination of crypts in neonatal chimaeric intestine: mixed crypts in neonatal intestine would suggest formation from multiple progenitors, whereas monoclonal crypts would suggest a single progenitor from the outset.

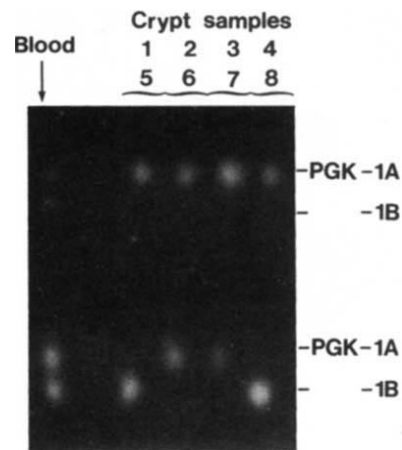


Fig. 3 Analysis of individual colonic crypts from PGK-1A/PGK-1B mosaic mice. The figure shows a representative gel from an analysis of 312 isolated colonic crypts (see below). The gel contains two rows of samples, upper and lower. The left-hand slot in each row contains a blood sample (PGK-1A/PGK-1B 50:50). The remaining four slots in each row each contain a single crypt sample, corresponding to either PGK-1A (six crypts) or PGK-1B (two crypts): none is mixed. Titration against known dilutions of a standard PGK-1A liver sample indicated that a contribution of a minority PGK type to a crypt would be detectable in this assay if it represented at least $\frac{1}{3}$ of the total, or ~ 40 cells.

Methods. Female mice heterozygous for the X-linked variant electrophoretic isoenzymes, PGK-1A and PGK-1B, were obtained from a mating between females homozygous for *Pgk-1^b*, and *Pgk-1^a* males⁷. Two 6-week-old females were killed by cervical dislocation, and a length (2 cm) of descending colon dissected out, flushed with PBS, opened longitudinally and incubated in EDTA (7.5 mg ml^{-1} in PBI buffer¹⁴, pH 8.2) at 37 °C for 1 h to loosen the attachment between the epithelium and underlying tissue. The colon was then transferred to ice-cold PBI for dissection. Pieces of epithelium containing between 10 and 100 crypts were isolated from different areas of the colon, dissected free and transferred to a watch-glass in cold PBI. Clumps were separated by gentle pipetting, and single crypts were taken up and sealed in microcapillaries in 1 μ l PBI for electrophoretic analysis. The crypts were freeze-thawed three times in liquid nitrogen, centrifuged, and supernatants analysed by Cellolog electrophoresis by the method of Bucher *et al.*¹⁵. The failure of 36/312 (11.5%) of the dissected crypts to give a band in the gel was caused by the occasional adherence of a crypt to the microcapillary wall.

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- Potten, C. S. & Hendry, J. H. in *Stem Cells* (ed. Potten, C. S.) 155-199 (Churchill Livingstone, Edinburgh, 1983).
- Trier, J. S. & Moxey, P. C. *Ciba Fdn Symp.* 70, 3-29 (1979).
- Eastwood, G. L. & Trier, J. S. *Anat. Rec.* 179, 303-310 (1974).
- Ponder, B. A. J., Wilkinson, M. M. & Wood, M. J. *Embryol. exp. Morph.* 76, 83-93 (1983).
- Ponder, B. A. J., Wilkinson, M. M., Wood, M. & Westwood, J. J. *Histochem. Cytochem.* 31, 911-919 (1983).
- Ponder, B. A. J. & Wilkinson, M. M. *Dev. Biol.* 96, 535-541 (1983).
- McMahon, A., Foster, M. & Monk, M. J. *Embryol. exp. Morph.* 74, 207-220 (1983).
- Cheng, H. & Leblond, C. P. *Am. J. Anat.* 141, 503-520 (1974).
- Oi, V., Jones, P. P., Goding, J. W., Herzenberg, L. A. & Herzenberg, L. A. *Curr. Topics Microbiol. Immun.* 81, 1-5 (1978).
- Etzler, M. *Enzymology* 28, 340-344 (1972).
- Schmidt, G. H., Ponder, B. A. J. & Wilkinson, M. M. *Anat. Rec.* 210, 407-411 (1984).
- Whittingham, D. G. & Wales, R. G. *Aust. J. Biol. Sci.* 22, 1065-1068 (1969).
- Schmidt, G. H., Garbutt, D., Wilkinson, M. M. & Ponder, B. A. J. *J. Embryol. exp. Morph.* (in the press).
- Hutchinson, H. T. *J. theor. Biol.* 38, 61-79 (1973).
- Whittingham, D. G. & Wales, R. G. *Aust. J. Biol. Sci.* 22, 1065-1068 (1969).
- Bucher, T., Bender, W., Fundele, R., Hofner, R. & Linke, I. *FEBS Lett.* 115, 319-324 (1980).

Generation of chick skeletal muscle cells in groups of 16 from stem cells

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The commonly accepted hypothesis explaining the control of skeletal muscle differentiation is that all myogenic precursor cells are equivalent and that they differentiate into post-mitotic muscle cells in response to exogenous signals, specifically low mitogen concentrations^{1,2}. Large clones derived from vertebrate myogenic cells, however, consist both of cycling precursors and of terminally differentiated, post-mitotic muscle cells³⁻⁷. Here, we count the total number of cells and the number of terminally differentiated cells (or nuclei, in fused cells) in large myogenic clones. The number of terminally differentiated cells per clone was usually equal to or just below a multiple of 16. This finding is not expected from a model postulating a homogeneous population of muscle precursor cells. Rather, our results suggest that a self-renewing stem cell exists in the skeletal muscle lineage. This cell can generate committed precursors which then give rise to cohorts of 16 terminally differentiated muscle cells. This model of myogenesis provides a simple explanation for the protracted and asynchronous nature of muscle differentiation in vertebrate embryogenesis.

We reported previously that small myogenic clones tend to contain 1, 2, 4, 8 or 16 terminally differentiated cells and no precursors⁷⁻¹⁰. These findings suggested that the final stages of the chick myogenic lineage consisted of a series of at least four determined, symmetrical cell divisions. We also found that myogenic clones of >16 cells were composed usually of a mixture of precursors and terminally differentiated cells, suggesting that an asymmetrical or stem cell-like division precedes the final four divisions⁷. We seek here to test this hypothesis. If an asymmetrical division occurred, the end cells should appear in numbers which are multiples of some power of two in the larger colonies. The numerical value of that power of two would signify the point in the cell lineage at which the cells became committed to terminal differentiation.

Cells were dissociated from the pectoral muscles of 14-day-old chick embryos as described previously⁷. Clones were generated by seeding 50 cells onto gelatin/fibronectin-coated 60-mm tissue culture dishes in 3 ml fresh medium. At this density, <2% of colonies arise from more than one cell. Immunocytochemical staining with anti-M-type creatine kinase (MCK) was used to detect post-mitotic, terminally differentiated muscle cells^{8,9}. Cultures were fixed after 4-7 days and stained with anti-MCK^{9,11} by the peroxidase/anti-peroxidase method¹². Almost all large (>16 cell) myogenic clones which had arisen were identified as small brown spots on the dishes. Peroxidase-stained colonies were placed on a trans-illuminating device and a 'map', containing the outline of each cell in the clone, was traced on paper to ensure accurate cell counts. Individual cells in the clones were then examined using phase and bright-field microscopy to determine the total number of cells (or nuclei, if fused cells were present) and the number of MCK⁺ cells (or nuclei, in the MCK⁺, multinucleated myotubes) in each clone. Only clones in which both the exact number of cells (nuclei) could be determined and every cell could be scored clearly as MCK⁺ or MCK⁻, were used for analysis. Approximately two-thirds of the generated clones were not suitable for analysis by these stringent criteria, principally because of multilayering and lobation of nuclei in some of the fused myotubes¹³, so that the exact number of nuclei could not be determined. This was an unavoidable

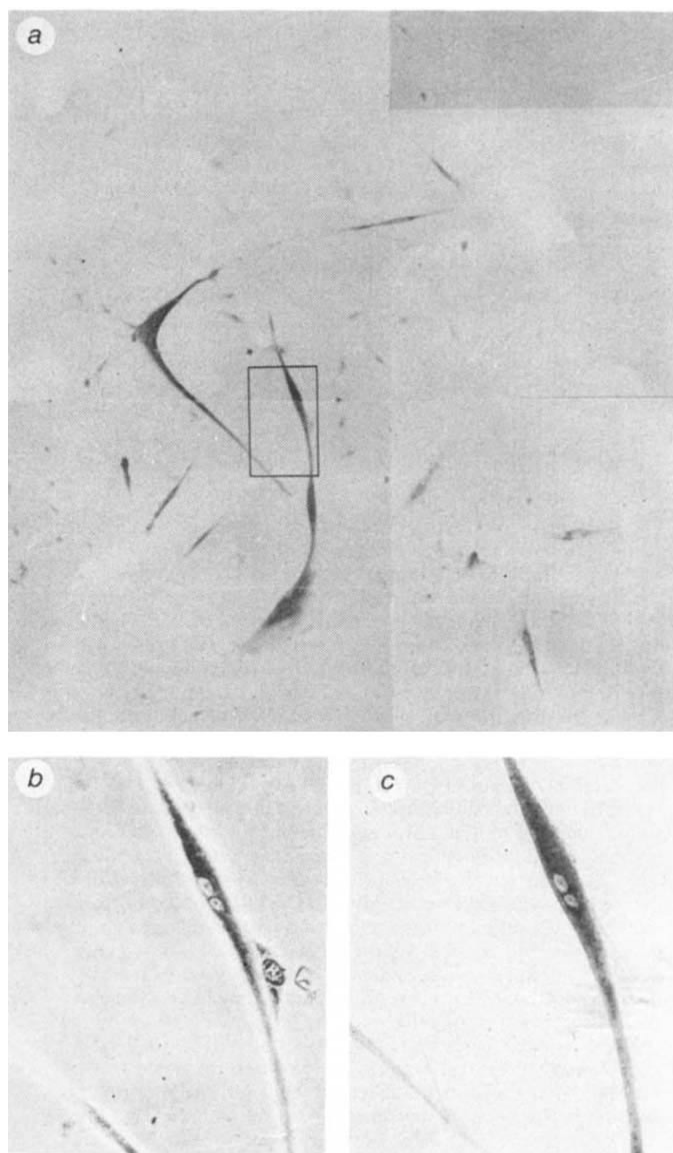


Fig. 1 Example of a large myogenic clone (clone R in Table 1), containing 80 MCK⁺ cells (nuclei) and 104 MCK⁻ cells. *a*, Composite low-power micrograph of entire clone; *b*, higher-power phase micrograph of area within rectangle; *c*, bright-field micrograph of same field, showing closely applied but clearly distinguishable MCK⁺ and MCK⁻ cells.

logistic restraint, but to prevent possible bias, selection of clones for analysis was made before any counts were performed. Also, counting of cells in the clones was 'blind' in the sense that a running total of cells was not kept. Instead, the number of nuclei in individual MCK⁺ and MCK⁻ profiles on the clone maps were counted and were added up after all the profiles in the clone had been analysed. An example of a large muscle colony containing MCK⁺ and MCK⁻ cells is shown in Fig. 1.

Table 1 shows the data obtained from an analysis of 24 clones. In eight cases (clones F, G, H, O, R, S, U and X), the number of terminally differentiated (MCK⁺) cells was precisely an integer multiple of 16. In all but two of the remaining cases, the number of MCK⁺ cells was closer to the next-highest multiple of 16 than to the next-lowest; in one case (clone I), the number was equidistant (Fig. 2). A model of myogenesis postulating a homogeneous population of precursor cells, in which 'commitment' to terminal differentiation occurs stochastically and without further cell division^{1,2}, would predict a uniform random distribution of these data. The observed distribution differs significantly from uniform random ($P < 0.001$, χ^2 test),

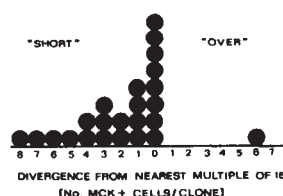


Fig. 2 Histogram showing divergence in number of MCK⁺ cells per clone from the nearest multiple of 16. Each circle represents one clone. For example, a clone which contained 33 MCK⁺ cells would be 'over' the nearest multiple of 16 by one cell; a clone containing 31 MCK⁺ cells would be one cell 'short' of the nearest multiple of 16.

however, and is therefore not consistent with such a model. The number of MCK⁺ cells in the clones and the total number of cells in the clones was not significantly different from uniform random distributions by the same analysis. The one-tailed appearance of the curve in Fig. 2 is also evidence against a microenvironmental induction mechanism for terminal differentiation (that is, that groups of about 16 precursor cells facilitate co-commitment to terminal differentiation); this model would predict a two-sided or gaussian curve with a modal value of $16n$. Instead, the simplest explanation for the observed data is that, after committing to terminal differentiation, muscle precursor cells 'count' exactly four cell divisions before becoming post-mitotic muscle end cells. Cases in which the number of MCK⁺ cells in the clone was not precisely, but just short of, a multiple of 16, can be attributed to cell death, or to mitotic asynchrony within cohorts of committed precursor cells; that is, the clones were fixed before all the cells in a given committed cohort had finished dividing. In support of this explanation, the data in Fig. 2 fit an exponential curve (Kolmogorov-Smirnov test). Similarly, if the number of divisions before terminal differentiation were loosely controlled (for example, a dilution or time model after the commitment step), then a two-sided distribution would again have been expected. In contrast, the one-sided appearance of the data provide strong support for a strict cell-cycle-dependent timing mechanism in myogenesis.

Although our data do not formally disprove the possibility that in some cases, committed cells gave rise to muscle cells after only three divisions, the statistical evidence suggests that this was at most a rare event. These observations confirm and extend earlier studies in which small myogenic clones tend to contain 2^n ($n = 0, 1, 2, 3, 4$) terminally differentiated cells⁷⁻¹⁰. We therefore suggest that, after commitment, a simple counting mechanism is the simplest interpretation of the data in Fig. 2.

We also found no consistent relationship between the number of MCK⁺ cells in the clones and the total number of cells in the clones; rather, the percentage of MCK⁺ cells in each clone varied considerably (Table 1). For example, in two cases (clones F and G), the clones contained 32 MCK⁺ cells and no MCK⁻ cells. By comparison, clone H also contained 32 MCK⁺ cells, but these comprised only 8% of the cells in the clone. These findings indicate that the stem-cell-like divisions in the myogenic lineage are not invariably asymmetrical, always resulting in the production of one cell committed to the four terminal divisions and one stem cell. Rather, they support the model illustrated in Fig. 3, incorporating both symmetrical and asymmetrical stem cell divisions. Once generated, a committed cell would invariably undergo four symmetrical divisions before terminal differentiation. Our data do not imply that any theoretical importance should necessarily be attributed to this particular number of cell divisions, but simply that the final divisions seem to be symmetrical and to be involved in the timing mechanism for terminal differentiation. It is conceivable that in different species, other embryonic stages and/or locations in the chick embryo and perhaps even in different culture conditions, the number of symmetrical divisions between commitment and terminal differentiation might be different (see ref. 7).

Myogenesis has long been considered a model system for studying vertebrate cell differentiation. The induction model is often cited as evidence for the instructive role of soluble factors in determining cell fate^{2,14-16}. This model has tended to focus genetic and biochemical studies of muscle differentiation onto the terminal phases of myogenesis, when it is assumed that all the regulatory events controlling muscle gene expression take place¹⁷. The induction model of myogenesis, however, has been

Table 1 Differentiation and proliferation data for myogenic clones

Clone	No. of MCK ⁺ cells	Divergence of no. MCK ⁺ cells from nearest multiple of 16*	Nearest multiple of 16	Total no. of cells	% MCK ⁺	Days in culture
A	13	-3	1	43	30	4
B	15	-1	1	111	14	4
C	27	-5	2	151	18	5
D	30	-2	2	93	32	6
E	30	-2	2	151	20	5
F	32	0	2	32	100	6
G	32	0	2	32	100	7
H	32	0	2	398	8	6
I	40	±8	2-3	102	39	6
J	57	-7	4	96	59	6
K	60	-4	4	119	50	6
L	61	-3	4	79	77	5
M	61	-3	4	91	67	6
N	63	-1	4	159	40	6
O	64	0	4	118	54	6
P	76	-4	5	111	69	5
Q	79	-1	5	169	47	6
R	80	0	5	184	44	7
S	80	0	5	174	46	6
T	106	-6	7	328	32	6
U	112	0	7	188	60	5
V	127	-1	8	332	38	6
W	134	+6	8	335	40	6
X	144	0	9	292	49	6

* Column lists difference, in number of cells, between the number of MCK⁺ cells observed and the nearest multiple of 16. Thus, a clone containing 33 MCK⁺ cells would be listed as +1 (rather than -15); a clone of 31 MCK⁺ cells would be listed as -1; a clone of 40 MCK⁺ cells would be ±8. (These data are also illustrated in Fig. 2.)

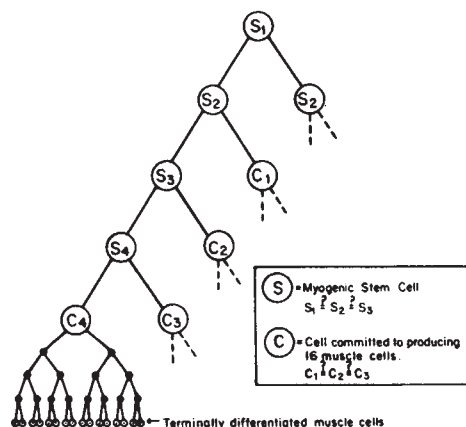


Fig. 3 Model for the avian myogenic lineage, illustrating the types of divisions which muscle stem cells can undergo. Symmetrical, producing two more stem cells; asymmetrical, producing one stem cell and one 'committed' precursor; terminal symmetrical, producing two committed cells. Committed cells in the chick undergo four symmetrical divisions to produce cohorts of 16 terminally differentiated muscle cells. Mechanisms controlling direction of stem cell divisions are unknown.

unsuccessful in explaining a number of observations on skeletal muscle development both *in vitro* and *in vivo*. Specifically, it does not explain the asynchronous time course of terminal muscle differentiation in a single area of developing muscle tissue¹⁸, in mass cultures^{19,20} and even a single clone³⁻⁷. To account for this asynchrony, a stochastic or probabilistic aspect has sometimes been added to the induction model^{2,21,22}. The results reported here do not support such a mechanism for myogenesis but instead strongly support a more deterministic, cell-cycle-dependent and step-wise model of myogenesis^{6-11,23,24}. Our model can account successfully for the protracted nature of muscle differentiation both in culture and in the embryo. If environmental factors control the number of muscle precursor or end cells, such factors may exert their influence in two ways, by affecting either the type of division made by the stem cell or the rate of progress through the succeeding four compartments of the lineage. This model also raises the interesting possibility that genetic or cytoplasmic regulatory events controlling muscle gene expression in the end-stage cells may occur at the stem cell level. Our findings also advise caution in extrapolating normal control mechanisms from the properties of myogenic cell lines which, unlike normal myogenic cells^{7,11,20}, can be induced to differentiate synchronously^{25,26}.

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- Konigsberg, I. R. *Dev Biol.* **26**, 133-152 (1971).
- Konigsberg, I. R. in *Pathogenesis of the Human Muscular Dystrophies* (ed. Rowland, L.P.) 779-798 (Elsevier, Amsterdam, 1977).
- Konigsberg, I. R. *Science* **140**, 1273-1284 (1963).
- Linkhart, T. A. & Hauschka, S. D. *Dev Biol.* **69**, 529-548 (1979).
- Hauschka, S. D. *Dev Biol.* **37**, 329-344 (1978).
- Abbott, J., Schiltz, J., Dienstman, S. & Holtzer, H. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1506-1510 (1974).
- Quinn, L. S., Nameroff, M. & Holtzer, H. *Expl Cell Res.* **154**, 65-82 (1984).
- Kligman, D. & Nameroff, M. *Expl Cell Res.* **127**, 237-247 (1980).
- Quinn, L. S. & Nameroff, M. *Differentiation* **24**, 111-123 (1983).
- Quinn, L. S. & Nameroff, M. *Differentiation* **24**, 124-130 (1983).
- Quinn, L. S. & Nameroff, M. *Differentiation* **26**, 112-120 (1984).
- Robinson, M., Quinn, L. S. & Nameroff, M. *Differentiation* **26**, 112-120 (1984).
- Sternberger, L. A. *Immunocytochemistry* (Wiley, New York, 1979).
- Capers, C. R. *J. biophys. biochem. Cytol.* **7**, 559-566 (1960).
- Caplan, A. L., Fiszman, M. Y. & Eppenberger, H. M. *Science* **221**, 921-927 (1983).
- Houseman, D. & Levenson, R. *Cell* **25**, 5-6 (1981).
- Alberts, B. et al. *Molecular Biology of the Cell* (Garland, New York, 1983).

- Devlin, R. B. & Emerson, C. P. *Cell* **13**, 599-611 (1978).
- Holtzer, H., Marshall, J. M. & Finck, H. *J. biophys. biochem. Cytol.* **3**, 705-723 (1957).
- Okazaki, K. & Holtzer, H. *J. Histochem. Cytochem.* **13**, 726-739 (1965).
- Yeoh, G. C. T. & Holtzer, H. *Expl Cell Res.* **104**, 63-78 (1977).
- O'Neill, M. C. *Dev Biol.* **53**, 190-205 (1976).
- Devlin, B. H., Merrifield, P. A. & Konigsberg, I. R. in *Muscle Development. Molecular and Cellular Control* (eds Pearson, M. L. & Epstein, H. F.) 355-366 (Cold Spring Harbor Laboratory, New York, 1982).
- Dienstman, S. R. & Holtzer, H. in *Results and Problems in Cell Differentiation* (eds Reinert, J. & Holtzer, H.) 1-25 (Springer, New York, 1975).
- Holtzer, H. in *Stem Cells and Tissue Homeostasis* (eds Lord, B. I., Potten, C. S. & Cole, R. J.) 1-28 (Cambridge University Press, 1978).
- Nadal-Ginard, B. *Cell* **15**, 855-864 (1978).
- Linkhart, T. A., Clegg, C. H. & Hauschka, S. D. *Dev Biol.* **86**, 19-30 (1981).

Evidence for polarization of plasma membrane domains in pancreatic endocrine cells

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Polarization of plasma membrane domains is an essential feature of secretory epithelial cells from exocrine glands. The surface of exocrine cells (a typical example is the acinar cell of the pancreas) is separated into an apical domain, where secretion occurs by exocytosis, and a basolateral domain, which senses variations of the internal milieu and is enriched with receptors for various hormones and secretagogues. It is unknown whether secretion is polarized in endocrine cells (except for thyroid follicular cells, which are organized into cavitory structures). To determine whether distinct plasma membrane domains exist in endocrine cells, we infected monolayer cultures of pancreatic endocrine cells with enveloped RNA viruses known to bud selectively from either the apical or basolateral domain in polarized epithelial cells¹. This asymmetrical budding is thought to reflect the polarized nature of the infected cells, as in non-polarized cells such as fibroblasts, the same viruses bud nonselectively from the entire cell surface¹. We show here that influenza virus and vesicular stomatitis virus (VSV) emerge asymmetrically from cultured pancreatic islet cells; this represents the first evidence for polarization of plasma membrane domains in pancreatic endocrine cells.

In monolayer cultures of endocrine pancreas infected with VSV or influenza virus, extensive virus budding was observed 3-5 h after infection with VSV and 8-9 h after infection with influenza virus. Viruses budding from the cell surface were identified by the continuity of the viral envelope with the host cell plasma membrane. This continuity allowed us to distinguish between budding viruses and viruses adsorbed to the cell surface

Table 1 Percentage of viruses budding from either the apical or basal plasma membrane domain in cultured insulin-producing cells

	Apical	Basal
Influenza virus*	93.93 ± 0.80‡	6.07 ± 0.80
VSV†	12.11 ± 4.16‡	87.88 ± 4.16

The apical domain was defined as the portion of the cell surface directly exposed to the culture medium, and the basal domain as the portion of the cell surface resting on the bottom of the dish. For the sake of rigorous quantitation, we analysed only the cells in the regions of the culture that were strictly monostriated. Although the cells satisfying this requirement represented a limited fraction (~30%) of the total cell population, qualitative observations in non-monolayer (usually bistratified) regions of the culture also showed polarized virus budding. Moreover, we counted only those viruses showing a clear continuity with the cell surface in the plane of the section. The results are the mean ± s.e.m. of four separate experiments.

* 8.5 h; † 4.5 h after infection.

‡ $P < 0.001$ compared with basal values; Student's *t*-test.

Fig. 1 Thin section perpendicular to a monolayer of pancreatic endocrine cells infected with VSV. *a*, Numerous VSV particles, appearing as dark spots at this low magnification ($\times 6,000$), are seen beneath the basal surface of the cells, but are virtually absent from the apical surface. *b*, Higher magnification ($\times 26,000$) of the area outlined by the rectangle in *a*. The arrow indicates a virus caught in the process of budding from the basal plasma membrane. SG, insulin-containing secretory granules.

Methods. Monolayer cultures of pancreatic islet cells from 1–3-day-old Wistar rats were established in 35-mm Falcon plastic Petri dishes as described previously² and used after 8 days of culture. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum (HINCS), 400 U ml⁻¹ sodium penicillin and 16.7 mM glucose. The cultures were infected for 1 h at 37 °C with VSV (MVOD and Summers Indiana Strain, given by Dr L. Roux) at a dose of 10⁸ plaque-forming units (PFU) per Petri dish in phosphate-buffered isotonic saline (PBS) containing 2 mg ml⁻¹ of bovine serum albumin (BSA). After infection, the cells were washed with PBS + 2 mg ml⁻¹ BSA and further incubated for 4.5 h at 37 °C in DMEM supplemented with 0.1% HINCS and 1 mg ml⁻¹ BSA. At the end of the incubation, the cells were washed again in PBS and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After washing in cacodylate buffer, the cultures were postfixed in 1% osmium tetroxide, stained *en bloc* with 2.5% uranyl acetate in 50% ethanol, dehydrated in graded ethanols and embedded *in situ* in Epon 812. Thin sections were cut perpendicular to the culture plane, stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

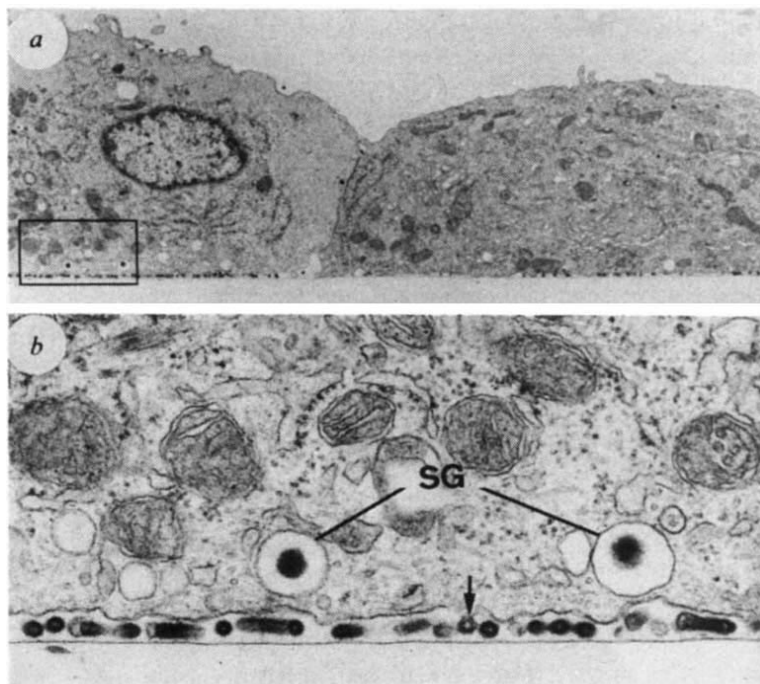
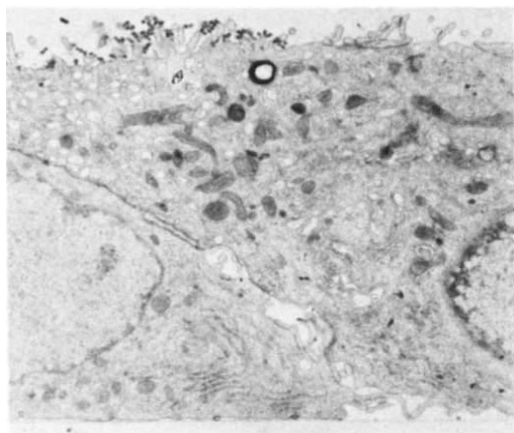


Fig. 2 Thin section perpendicular to a monolayer of pancreatic endocrine cells infected with influenza virus. Extensive budding of the virus is seen on the apical surface. In contrast, the basolateral surface is virtually devoid of budding viruses, $\times 6,300$.

Methods. The cultures were infected for 1 h at 37 °C with the A/WS (1933, H₁N₁) strain of influenza virus (given by Dr Paccaud) at 10⁸ PFU per Petri dish in DMEM + 2 mg ml⁻¹ BSA. After infection, the cells were washed with DMEM and further incubated for 8.5 h at 37 °C in DMEM + 2% HINCS. At the end of the incubation, the cells were fixed and processed for electron microscopy as described in Fig. 1 legend.



or entering the cell by receptor-mediated endocytosis (a very rare event at the time points studied). In cultures infected with VSV, a large number of virions were seen between the basal surface of the endocrine cells and the bottom of the culture dish (Fig. 1). Some of these virions were caught in the process of budding (Fig. 1*b*), whereas others appeared free in the extracellular space. Only in rare instances did we observe VSV particles emerging from the apical surface of islet cells (Fig. 1*a*). In contrast, in cultures infected with influenza virus, extensive budding was seen on the apical surface of the endocrine cells, whereas the basolateral surface appeared virtually devoid of budding viruses (Fig. 2).

To quantitate these observations, we counted the number of viruses budding from either the apical or basal surface of the islet cells in four distinct experiments; only those viruses showing a clear continuity with the cell surface were included. We found that over 90% of influenza viruses emerged from the apical domain of the islet cell plasma membrane, while 88% of vesicular stomatitis viruses budded from the basal surface (Table 1). Although the quantitation was done on insulin-producing β cells (by far the most frequent cell type in the cultures), qualitative observations on the other islet cell types (α cells and D cells) also revealed asymmetry of viral budding. In contrast to

islet cells, however, polarized budding was not seen in the fibroblasts neighbouring the endocrine cell clusters: in the fibroblasts, both viruses emerged indiscriminately from both the adherent and free surface of the cell (data not shown).

These experiments demonstrate that the influenza virus and VSV emerge asymmetrically from islet cells as they do in polarized epithelia¹. Previous studies with MDCK kidney-derived cells have shown that the polarity of viral budding is a consequence of the asymmetrical insertion of the viral envelope glycoproteins into distinct plasma membrane domains². Thus, our results suggest that cultured pancreatic endocrine cells have the ability to sort out and target different viral proteins to specific plasma membrane domains. As it is believed that viral glycoproteins are treated by the cell in the same way as cellular proteins, this sorting ability of islet cells probably extends to endogenous membrane proteins.

Enveloped viruses are becoming powerful tools for the biochemical analysis of specific regions of the cell membrane, as the lipid composition of the viral envelope reflects that of the plasma membrane from which it is derived³. By isolating the viruses budding from either the apical or basolateral surfaces of pancreatic endocrine cells, it should be possible, therefore, to collect selected samples of these membrane domains and to

characterize their lipid composition, as has been done with MDCK cells³.

Separation of the cell surface into distinct membrane domains is an essential feature of secretory epithelial cells in exocrine tissues, but has not yet been demonstrated in endocrine cells (except for thyroid follicular cells, as mentioned above). It is unknown, for example, whether hormone release occurs through the same region of the cell surface that receives exogenous stimuli or whether endocrine cells possess distinct 'sensitive' and 'effector' poles. The data presented here clearly demonstrate polarization of plasma membrane domains in cultured pancreatic endocrine cells and provide the basis for future efforts to unmask plasma membrane polarization in endocrine cells *in vivo*. In this respect, it is worth mentioning that recent physiological data are consistent with the possibility that distinct venous (receptor-rich) and arterial (secretory) poles exist in pancreatic insulin-producing cells⁴.

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1. Rodriguez-Boulan, E. & Sabatini, D. D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5071-5075 (1978).
2. Rodriguez-Boulan, E. & Pendergast, M. *Cell* **20**, 45-54 (1980).
3. Van Meer, G. & Simons, K. *EMBO J.* **1**, 847-852 (1982).
4. Kawai, K., Ipp, E., Orci, L., Perrelet, A. & Unger, R. H. *Science* **218**, 477-478 (1982).
5. Lambert, A. E., Blondel, B., Kanazawa, Y., Orci, L. & Renold, A. E. *Endocrinology* **90**, 239-248 (1972).

Calcium regulation of molluscan myosin ATPase in the absence of actin

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In the myosin-linked regulatory mechanism typified by the molluscan scallop adductor muscle, contraction is controlled by Ca^{2+} binding to sites on the thick filament protein, myosin¹. The regulatory light chains of myosin heads are involved directly in this mechanism and early studies^{2,3} suggested that, in the absence of Ca^{2+} , these subunits prevent the interaction of a myosin-adenosine nucleotide complex with the actin-containing thin filament. Subsequently, Ashiba *et al.*⁴ reported that the steady-state ATPase of molluscan myosin exhibits a limited degree of Ca^{2+} activation in the absence of actin. Recently, however, we have shown that steady-state ATPase activity in relaxing conditions is dominated by the unregulated molecules in the myosin preparation⁵. Single-turnover kinetic methods are required to monitor the highly suppressed ATPase activity of the regulated population. Using the latter approach, we report here that scallop myosin ATPase is reduced about 100-fold on removal of Ca^{2+} . The regulatory light chains maintain the relaxed state via conformational changes which suppress the product release steps, irrespective of the presence of actin.

The actin-activated Mg^{2+} -ATPase activity of scallop heavy meromyosin (HMM), a double-headed proteolytic subfragment of myosin, can be followed directly by turbidometry. When a near-stoichiometric concentration of ATP is added to acto-scallop HMM, a rapid drop in turbidity occurs due to dissociation of the protein complex, followed by a recovery as the ATP is exhausted and the rigor state is reformed. In the absence of Ca^{2+} , this recovery phase is markedly biphasic (ref. 5 and Fig. 1a). We have interpreted this in terms of a heterogeneous HMM population, in which an unregulated fraction (typically 20-30%) hydrolyses ATP rapidly, leaving the regulated molecules to undergo a slow single turnover with a half-time of several minutes (rate = $4.5 \times 10^{-3} \text{ s}^{-1}$, Fig. 1a). This very slow rate for the Ca^{2+} -free actin-activated ATPase implies that actin-free HMM ATPase should be at least as slow in the same relaxing conditions. Although typical steady-state rates for the HMM ATPase alone are 0.3 s^{-1} (+ Ca^{2+}) and 0.1 s^{-1} (- Ca^{2+}), the

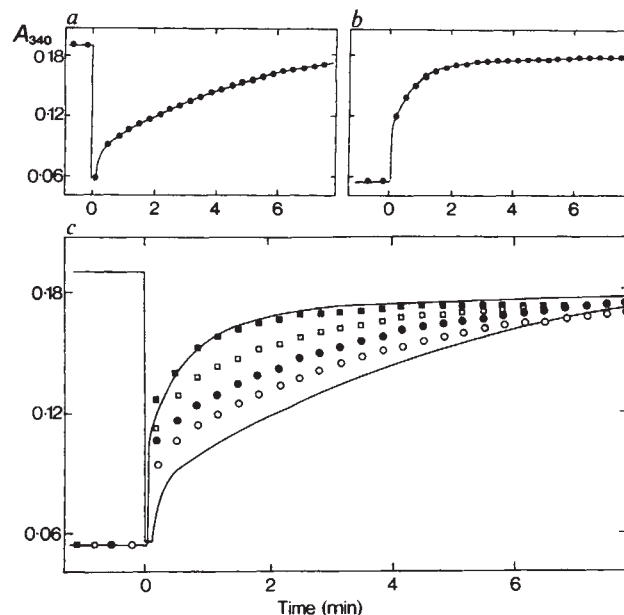


Fig. 1 The rate of actin binding to scallop (*Pecten maximus*) HMM, in the absence of Ca^{2+} and presence of nucleotide, was recorded by following the turbidity changes at 340 nm, using a Perkin-Elmer LS5 spectrophotometer. These experiments were carried out in 10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid (TES), 20 mM NaCl, 5 mM MgCl_2 , 100 μM EGTA, pH 7.5, at 20 °C and using 2 μM actin, 2 μM HMM (myosin head concentration) and 10 μM nucleotide. (Note that although a five-fold molar excess of ATP over HMM was used, the slow phases of the observed profiles are single turnovers of the regulated HMM, for reasons discussed previously⁵.) *a*, Actin was pre-mixed with HMM to give a solution of high turbidity. Addition of ATP at time 0 caused a rapid dissociation of the acto-HMM followed by a biphasic recovery, as noted previously⁵. *b*, The profile obtained on addition of actin at time 0 to a preincubation mixture of HMM with ADP. When this experiment was repeated in the presence of Ca^{2+} , the turbidity increase was >90% complete the manual mixing time. The records from *a* and *b* are also shown in *c* (solid lines) and represent the two extreme profiles of the following actin chase experiment. HMM was premixed with ATP and then actin was added to aliquots of this mixture in separate cuvettes in an autochanger attachment. Actin was added at: $\circ$, 0.85 min; $\bullet$, 2.3 min; $\square$, 5.6 min; and $\blacksquare$, 21 min after addition of ATP to the HMM. To allow comparison of the turbidity profiles, time 0 refers to the point of actin addition. The jump in turbidity at this time is attributed to actin binding to the unregulated HMM population⁵, as are the rapid phases in *a* and *b*.

latter could be dominated by the contribution from unregulated (permanently activated) HMM molecules. We have therefore used limited turnover analysis to measure the ATPase activity of HMM in the absence of actin.

Initially, this problem was investigated indirectly by performing an actin chase experiment. HMM and ATP were pre-mixed at near-stoichiometric concentrations in the absence of Ca^{2+} and the progress of their reaction was followed by the changes in the turbidity profile on addition of actin at different time intervals of preincubation. The data shown in Fig. 1a, where HMM and ATP were mixed in the presence of actin, represent, in effect, an actin chase at zero time. When ATP was added to the HMM before the actin, the rate of the slow phase of the turbidity increase on actin addition was time-dependent. The observed turbidity change became progressively faster with longer preincubation times, reaching a maximum rate of 0.023 s^{-1} (Fig. 1c) comparable with that of the slow phase when actin bound to HMM in the presence of ADP (Fig. 1b). The actin chase experiment (Fig. 1c) revealed that the transition between the slowest (Fig. 1a) and the fastest (Fig. 1b) rate occurred with a half-time ($t_{1/2}$) of 2-3 min. A rate constant of $\sim 4.6 \times 10^{-3} \text{ s}^{-1}$

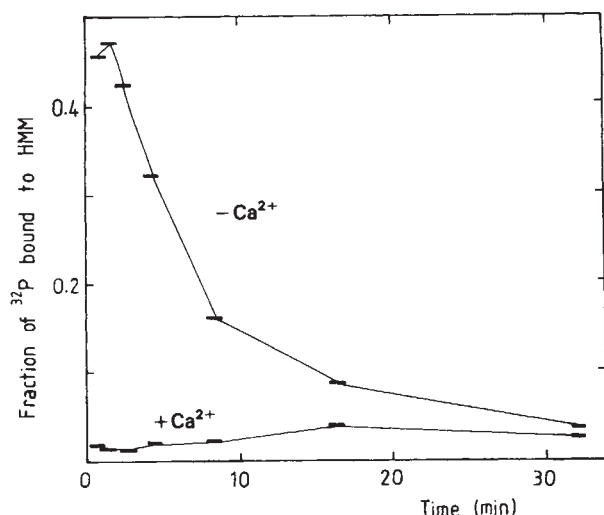


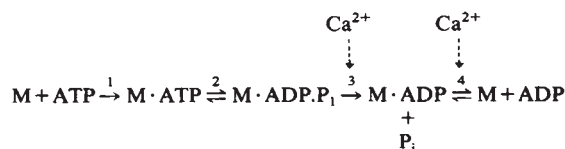
Fig. 2 The rate of P_i release from scallop HMM following ATP hydrolysis in the presence and absence of Ca^{2+} . HMM (17 μ M myosin heads) was incubated with [γ - 32 P]ATP (21 μ M) at 20 °C and pH 7.5 in 10 mM TES, 20 mM NaCl, 1 mM Mg^{2+} and either 100 μ M free Ca^{2+} or 100 μ M EGTA. Aliquots were removed at the times indicated and subjected to rapid separation on Sephadex G-50 by column centrifugation⁷ for 1 min (represented by the error bars on the x-axis). The radioactivity eluting with the protein is expressed as a percentage of the total counts applied to the column. The fraction of 32 P recovered with time from a similar experiment using subfragment 1, in the presence or absence of Ca^{2+} , was comparable with that observed with HMM in the presence of Ca^{2+} .

was obtained from an exponential fit of the turbidity at a time 2.5 min after actin addition, against the time of preincubation of HMM and ATP. We interpret this result in terms of the formation of a long-lived intermediate, when HMM is mixed with ATP in the absence of Ca^{2+} , which binds to actin only weakly. This intermediate is transformed slowly (in the case of regulated molecules) to M.ADP, the myosin-ADP equilibrium complex which remains at the end of the reaction and which can be generated by direct addition of ADP to HMM. In the presence of Ca^{2+} , ATP turnover was rapid and no slow phase of actin binding was observed in an actin chase experiment.

To characterize the ATPase pathway further, single turnover experiments were performed using [γ - 32 P]ATP to monitor the progress of the hydrolysis step and inorganic phosphate (P_i) release⁶. On quenching the reaction with perchloric acid after 15 s, we found that all (>95%) the ATP had been hydrolysed, both in the presence and absence of Ca^{2+} . When the unquenched reaction mixture was subjected to rapid separation on Sephadex G-50 by column centrifugation⁷, however, a significant fraction of the 32 P remained bound to the HMM in the absence, but not the presence, of Ca^{2+} (Fig. 2). Furthermore, the bound 32 P was released from the HMM with a rate constant of $2.1 \times 10^{-3} s^{-1}$ (obtained by a computer fit to an exponential). This experiment provides direct evidence for a slow step in the ATPase pathway of HMM alone and is not subject to the potential complications of the turbidity assay⁸. It was found also that the P_i undergoes

extensive oxygen exchange with solvent water during a single turnover of ATP (M. R. Webb, C. W. & C. R. B., unpublished observations), which implicates a rapid reversal of the hydrolysis step relative to P_i release⁶. We therefore identify the long-lived intermediate as M.ADP. P_i and assign the slow Ca^{2+} -sensitive step to P_i release.

A minimal scheme for the discussion of these results is represented by the following equation:



Step 2 is rapid and reversible irrespective of the Ca^{2+} concentration. Step 3 is rate-limiting and Ca^{2+} sensitive, with a rate, in relaxing conditions, of $2.1 \times 10^{-3} s^{-1}$ (Fig. 2). This increased by about 100-fold on Ca^{2+} activation to account for the overall steady-state rate of $0.3 s^{-1}$. Furthermore, Fig. 1b shows that ADP displacement by actin is rather slow ($0.023 s^{-1}$) in relaxing, but not activating, conditions. Although this experiment does not provide a direct measure of the rate of step 4, the results suggest that this step is also Ca^{2+} sensitive. This conclusion is supported by the finding that the ADP-induced fluorescence enhancement of scallop HMM is dependent on Ca^{2+} and we suggest that ADP binds more tightly to the regulated fraction of HMM in the absence of Ca^{2+} than in its presence¹³. Thus, although the overall pathway of the scallop myosin ATPase is similar to that of rabbit skeletal muscle myosin⁶, steps 3 and 4 are Ca^{2+} sensitive in the former case and display a considerable reduction in the magnitude of their rate constants in relaxing conditions. In these conditions, the observed steady-state rate of $0.1 s^{-1}$ suggests that there is a dominating contribution from an unregulated fraction (30%) in the scallop HMM preparation. Significantly, myosin subfragment 1 and HMM with a depleted light-chain content both exhibit Ca^{2+} -insensitive steady-state ATPase rates of $\sim 0.3 s^{-1}$ (ref. 13). Thus, the unregulated population in normal HMM preparations could be accounted for by molecules of this type.

In conclusion, our data show that in the absence of Ca^{2+} , fully regulated HMM ATPase activity is suppressed about 100-fold by inhibition of the P_i release step. This is achieved presumably through a conformational change involving the regulatory light chains and is independent of actin. Evidence for this change comes from the light-chain movements detected by crosslinking studies⁹ and the crossbridge disposition observed by electron microscopy of isolated scallop thick filaments¹⁰. Actin amplifies the Ca^{2+} sensitivity by further enhancing the ATPase in the presence of Ca^{2+} to give a total of >600-fold activation of the relaxed rate⁵. A marked inhibition of the ATPase in the absence of Ca^{2+} would account for the economy of ATP use in relaxed muscle *in vivo*¹¹. In contrast, such a high *in vitro* degree of suppression of the ATPase in the absence of Ca^{2+} has yet to be demonstrated for vertebrate skeletal muscle preparations, for which the existence of a myosin-linked regulatory system remains controversial¹².

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- Kendrick-Jones, J. & Scholey, J. M. *J. Muscle Res. Cell Motility* **2**, 347-372 (1981).
- Szent-Györgyi, A. G., Szentkirályi, E. M. & Kendrick-Jones, J. *J. molec. Biol.* **74**, 179-203 (1973).
- Marston, S. & Lehman, W. *Nature* **252**, 38-39 (1974).
- Ashiba, G., Asada, T. & Watanabe, S. *J. Biochem., Tokyo* **88**, 837-844 (1980).
- Wells, C. & Bagshaw, C. R. *FEBS Lett.* **168**, 260-264 (1984).

- Trentham, D. R., Eccleston, J. F. & Bagshaw, C. R. *Q. Rev. Biophys.* **9**, 217-281 (1976).
- Penefsky, H. S. *J. biol. Chem.* **252**, 2891-2899 (1977).
- Ando, T. & Scales, D. *Biophys. J.* **45**, 352a (1984).
- Hardwicke, P. M. D., Wallimann, T. & Szent-Györgyi, A. G. *Nature* **301**, 478-482 (1983).
- Craig, R. & Vibert, P. *Biophys. J.* **45**, 150a (1984).
- Ferenczi, M. A., Homsher, E., Simmons, R. M. & Trentham, D. R. *Biochem. J.* **171**, 165-175 (1978).
- Lehman, W. *Nature* **274**, 80-81 (1978).
- Wells, C., Warriner, K., Bennett, A. J. & Bagshaw, C. R. *Biochem. Soc. Trans.* (in the press).

Is sequence conservation in interferons due to selection for functional proteins?

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The human α -interferon (IFN- α) gene family consists of at least 14 potentially functional non-allelic members; the amino acid sequences they encode differ from each other by up to ~20% of their residues (ref. 1 and K. Henco *et al.*, in preparation). Human IFN- β , which is encoded by a single gene, is distantly related to the IFN- α family²; it differs in 67% of its residues from IFN- α_2 . There is considerable evidence that IFN- α and - β compete for the same receptors on their target cells³⁻⁶. Comparison of 14 non-allelic human IFN- α sequences and the IFN- β sequence has revealed that 37 of 166 residues are completely conserved and that several of these are arranged in clusters, for example at positions 29-33, 47-50 and 136-150. It is commonly held (for example, refs 2, 7) that evolutionary conservation of amino acids indicates that the residues in question are essential for function. To test this hypothesis in the case of IFNs, we have introduced single site-directed point mutations into the strictly conserved codons 48 and 49 of the IFN- α_2 gene which form part of the longest uninterrupted cluster (position 47-50). We report here that the mutant proteins, containing Tyr, Ser and Cys instead of Phe48, or His instead of Gln49, have biological activities indistinguishable from those of wild-type IFN- α . In addition, when Glu62, a residue conserved in all known α and β IFNs of man, mouse and cattle, was replaced by Lys, antiviral activity remained unchanged.

Site-directed point mutations were generated using four synthetic oligonucleotide primers containing single mismatches⁸⁻¹⁰ (Fig. 1a). In the expression plasmid used for mutagenesis and IFN production, pEMBL8(-)P α_2 (Fig. 1b), the mature human IFN- α_2 coding sequence was fused to the ribosome binding sequence of the 1.1 gene of phage T7 and placed under the control of the P₁ promoter of pBR322 (ref. 11). The plasmid also contained the 1,300-base pair (bp) intergenic fragment of phage f1, oriented such that superinfection with phage f1 yielded single-stranded, circular DNA¹² containing the IFN- α_2 (-) strand sequence. A mutagenic oligonucleotide was hybridized to the single-stranded template DNA and elongated with DNA polymerase in the presence of T₄ DNA ligase. The resulting double-stranded circular DNAs were introduced into *Escherichia coli*, and transformed colonies were transferred to filters and screened with the appropriate ³²P-labelled primer as probe^{13,14}.

Plasmid DNA recovered from positive colonies was resealed and subjected to restriction and sequence analysis. Figure 2 shows the relevant regions of the sequence gels of the four mutant and the wild-type IFN genes used to produce modified IFN in *E. coli* HB101. Interferons were partially purified (Fig. 3 legend) and aliquots analysed by SDS-polyacrylamide gel electrophoresis; the amount of IFN in each preparation was determined by scanning the stained gels and integrating the areas under the peaks, using known amounts of pure IFN- α_2 (a gift from M. Fountoulakis) as reference (Fig. 3). The IFN content of the partially purified preparations was 30-40%.

To confirm the amino acid substitutions, a sample of each IFN was purified by SDS-polyacrylamide gel electrophoresis and subjected to amino acid analysis. The mutant proteins showed the expected changes (Table 1); the number of Phe residues in the modified IFN- α_2 -A, -B and -C was reduced by

Fig. 1 Template and primers used for site-directed mutagenesis. A mismatch-containing oligonucleotide (a) was hybridized to a circular, single-stranded vector containing the IFN- α_2 (-) strand sequence (b), elongated with DNA polymerase and the newly formed DNA strand closed with DNA ligase. Double-stranded circular DNA was cloned in *E. coli* and colonies containing the mutated DNA were identified by hybridization to the mismatch-containing oligonucleotide. The oligonucleotides are aligned with the wild-type sequence of the IFN- α_2 gene around the region encoding the conserved amino acid cluster 47-50 (boxed). Mismatched bases and substituting amino acids are underlined. Abbreviations: Sh.D, ribosome binding sequence; Ap^r, ampicillin-resistance gene; P, P₁ promoter; F1(IG), f1 intergenic fragment.

Methods. a, Oligonucleotides were synthesized with the Applied Biosystems DNA synthesizer model 380A as specified by the supplier, purified by electrophoresis through a denaturing 12% polyacrylamide gel and chromatography on Sephadex G-25. Oligonucleotides A, B and C were synthesized as a mixture. b, The expression vector pEMBL8(-)P α_2 was prepared by joining the large *Pvu*II fragment of plasmid pEMBL8(-) (ref. 12) to a DNA segment (derived from plasmid pPIT α_2 , a gift from N. Panayotatos) containing the mature human IFN- α_2 coding sequence fused to the ribosome binding site of the 1.1 gene of phage T₇ (a 112-bp *Sall*/*Eco*RI fragment from plasmid pNKS97 (ref. 25)). The fusion was such that the initiation triplet directly preceded the TGT triplet encoding the first amino acid of mature IFN- α_2 ; the ribosome binding site was preceded by a 231-bp fragment derived from positions 2,804-3,035 of pBR322, containing the P₁ promoter¹¹. The IFN- α_2 insert was in the same orientation as the β -lactamase gene and therefore the single-stranded hybrid phage DNA derived from this plasmid contained the IFN- α_2 (-) strand sequence. To prepare hybrid phage, *E. coli* JM103 (ref. 26), containing pEMBL8(-)P α_2 , was superinfected with phage f1 (strain IR-1) and the resulting phage purified as described elsewhere¹². The single-stranded hybrid DNA was separated from contaminating f1 DNA (present in about threefold excess) by preparative agarose gel electrophoresis²⁷. Synthesis of double-stranded DNA using a mismatch-containing oligonucleotide as a primer was essentially as described elsewhere²⁸. One pmol (~1.5 μ g) of purified single-stranded pEMBL8(-)P α_2 DNA, 20 pmol 5'-³²P-labelled primer (~50,000 c.p.m.), 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 75 mM NaCl and 1 mM DTT (final volume, 10 μ l) were kept for 5 min at 70 °C and 1 h at 22 °C. The four deoxynucleoside triphosphates and ATP (in the same buffer as above, final concentration, 0.5 mM each), 200 U T₄ DNA ligase (Biolabs) and 2 U DNA polymerase I (Klenow fragment, Biolabs) were added (final volume, 20 μ l). After 20 h at 22 °C, the DNA was recovered by phenol extraction and ethanol precipitation, dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and fractionated on a 0.8% low gel temperature agarose gel containing 0.5 μ g ml⁻¹ ethidium bromide. The double-stranded circular DNA was recovered and cloned in *E. coli* JM103. Ampicillin-resistant colonies were transferred to Whatman 541 filters and processed as described by Taub and Thompson¹³ up to the phenol/chloroform treatment, washed three times for 3 min in 6 × NET (NET is 150 mM NaCl, 15 mM Tris-HCl pH 7.5, 1 mM EDTA), twice in 95% ethanol and air-dried. Pre-hybridization and hybridization were as described by Wallace *et al.*¹⁴. Plasmid DNA was extracted from positive colonies²⁹; monomeric DNA was isolated from low gel temperature agarose gels and resealed.

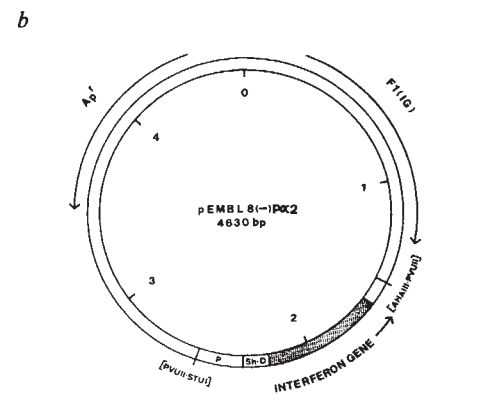
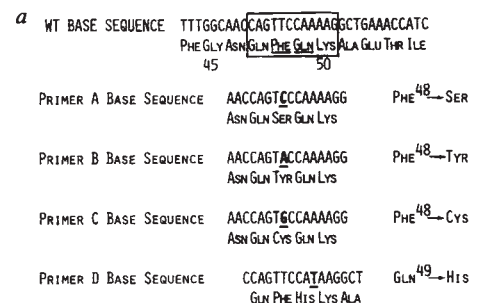


Fig. 2 Nucleotide sequence analysis of the coding sequence around codons 48–49 of wild-type and modified α_2 -interferons. From left to right, the sequence ladders of mutants D, C, B, A and wild type, respectively, are shown. The order of the lanes is G, A+G, T+C and C. Plasmid DNA was isolated²⁹, cut with *Bgl*III, 5'-³²P-labelled and sequenced by the Maxam and Gilbert procedure³⁰.

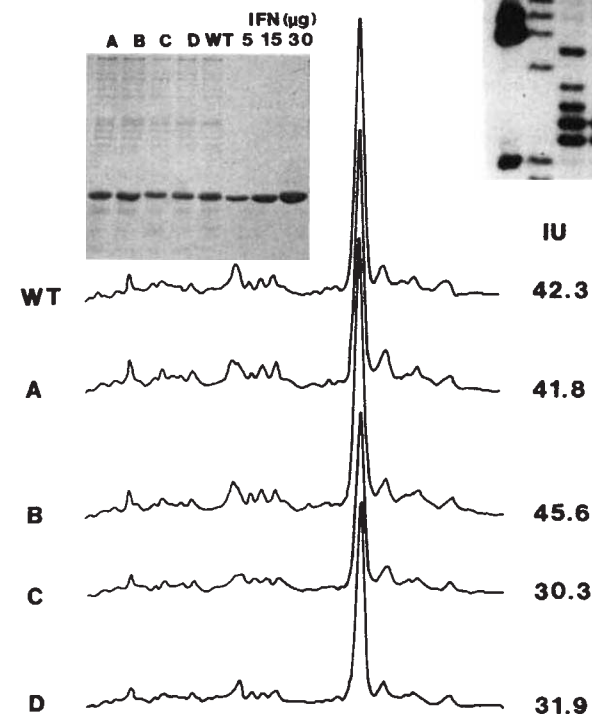
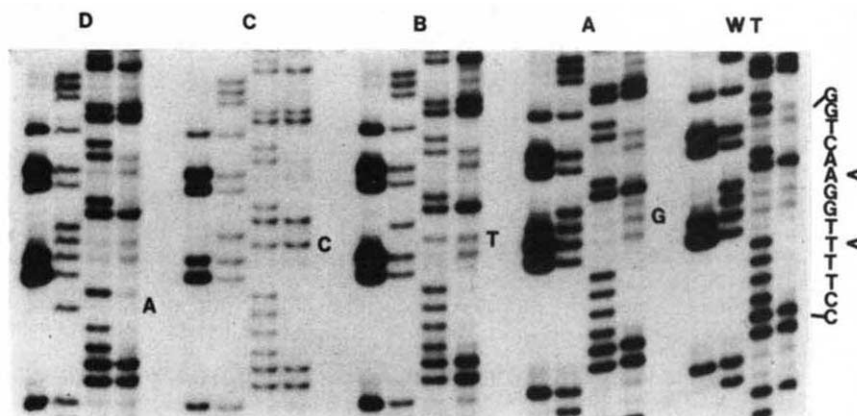


Fig. 3 Densitometric determination of the IFN content of purified preparations of wild-type and modified IFN from *E. coli*. Partially purified preparations of IFN (modified IFN- α_2 -A, -B, -C and -D, and wild-type IFN- α_2 (see Fig. 1)) were run through a polyacrylamide gel and the IFN content determined by measuring the area under the IFN peak relative to the area given by known quantities of homogeneous IFN- α_2 .

Methods. *E. coli* HB101 harbouring pEMBL8(-)P α_2 with the wild-type or the mutated IFN- α sequences were grown in 1-l cultures in L-broth in low aeration conditions at 37 °C to an A_{650} of about 1.0 and maintained in stationary condition for 30–40 h. Aliquots of the culture were lysed in urea-SDS buffer³¹ and tested for IFN activity by the cytopathic effect reduction assay on human WISH cells challenged by Mengo virus³². Each culture yielded $\sim 4 \times 10^8$ U IFN activity (measured relative to a laboratory reference sample that had been titrated against a NIH standard). The cells were collected by centrifugation, resuspended in 20 ml 10% sucrose, 0.1 M Tris-HCl (pH 7.5), 5 mM EDTA, 0.2 mM DTT and incubated with 3 mg lysozyme for 45 min at 22 °C. The cells were pelleted, resuspended in 20 ml of the above buffer without sucrose and subjected to four cycles of freezing and thawing. The insoluble pellet was washed twice with 20 ml phosphate-buffered saline (PBS) containing 2 M urea, 0.2 mM DTT, 10 mM benzamidine, and the IFN was extracted with 1.5 ml PBS containing 7.5 M guanidine-HCl, 0.5 mM EDTA and 0.2 mM DTT. The sample was diluted 20-fold with PBS containing 10 mM benzamidine, 1 mM EDTA, 0.2 mM DTT, clarified by centrifugation and concentrated to ~ 2 ml by ultrafiltration (Millipore CX-10 immiscible ultrafiltration units); recovery of IFN activity was 2.4 to 4×10^8 U (60–100%). Aliquots were electrophoresed through 17% polyacrylamide-SDS gels²² at 5–10 V cm^{-1} for 5–8 h alongside known amounts of highly purified IFN- α_2 (specific activity 1.5×10^8 U per mg; a gift from M. Fountoulakis). After staining with 0.25% Coomassie brilliant blue R250 in 50% methanol, 7.5% acetic acid, the gels were destained in 50% methanol, 7.5% acetic acid and scanned using a densitometer equipped with integrator (Shimadzu CS-930). The peak areas for 5, 15 and 30 μg of the standard IFN were 27.7, 58.6 and 96.0 integration units (IU), respectively. The inset shows the stained gel.

about 1 (0.6–1.2), as was the value for Glx in IFN- α_2 -D, and IFN- α_2 -A, -B and -D showed an increase by about one equivalent of Ser, Tyr and His, respectively. Cys (expected to change in IFN- α_2 -C) was not determined.

The four mutant IFNs were indistinguishable from wild-type IFN- α_2 (Table 2) with respect to their biological activities. The specific antiviral activities of the wild-type and the four modified IFNs were 1.1 – 1.9×10^8 U per mg on human WISH cells; there were also no significant differences when the activities were assayed on bovine MDBK or murine L929 cells. Moreover, all preparations had the same inhibitory effect on Daudi cells¹⁵, as determined by cell growth and ^3H -thymidine incorporation.

We had initially been interested in identifying residues which could be responsible for binding to the human IFN receptor. Some IFNs, in particular murine and human, are quite specific with respect to their target cells, and we therefore considered that human IFN- α s and - β (believed to act on the same receptor) would show a set of conserved amino acids which would overlap but not coincide with the set of conserved murine positions, and that positions conserved in man but not in mouse might be important for receptor binding. We subsequently generated a further mutation, Glu to Lys, in position 62, which is constant not only in all known human IFN- α s and IFN- β , but also in

Table 1 Amino acid analysis of modified interferons

	IFN	WT	A Phe $\rightarrow$ Ser	B Phe $\rightarrow$ Tyr	C Phe $\rightarrow$ Cys	D Gln $\rightarrow$ His
Asx	12	12.4	13.1	13.1	12.9	12.9
Thr	10	9.1	9.4	9.4	9.2	9.3
Ser	14	12.4	<u>14.5</u>	13.2	12.7	13.0
Glx	26	26.5	27.2	26.8	26.7	<u>25.9</u>
Gly	5	6.5	7.1	6.8	6.5	6.8
Ala	8	9.2	9.2	9.1	9.2	9.2
Val	7	6.9	6.9	7.0	7.1	6.9
Met	5	5.1	4.9	4.4	4.7	5.9
Ile	8	7.9	7.9	7.9	7.9	7.9
Leu	21	21	21	21	21	21
Tyr	5	5.1	5.1	<u>6.2</u>	5.1	5.2
Phe	10	9.9	<u>8.7</u>	<u>9.3</u>	<u>8.9</u>	10.1
Lys	10	10.1	10.1	10.2	10.1	10.2
His	3	3.0	3.1	3.1	3.0	4.0
Arg	10	10.2	9.9	9.6	11.0	9.9
Pro	5	6.9	7.0	5.8	6.9	6.8

Partially purified IFN (see Fig. 3 legend; 150–300 μg protein) was subjected to preparative SDS-polyacrylamide gel electrophoresis²² (15% acrylamide, 0.09% bis-acrylamide). Gels were lightly stained with Coomassie blue (30 s) and partially destained. The IFN-containing bands were excised, eluted into 2–3 ml 50 mM Na-phosphate, 2 mM dithiothreitol (DTT), 0.2% SDS or electroeluted into a buffer containing 50 mM NH_4HCO_3 , 0.2% SDS and 1 mM DTT and precipitated twice with trichloroacetic acid. The dried precipitates (50–100 μg of protein each) were subjected to acid hydrolysis and amino acid analysis²³. The tabulated values were normalized relative to the leucine content (21 U leucine per mol IFN- α_2). Underlined values indicate differences between wild type and mutant IFNs. WT, wild type.

Table 2 Biological activity of wild-type and modified IFNs

IFN preparation	Specific antiviral activity (U per mg)			Inhibitory activity (IFN concentration (pg ml ⁻¹) for 50% inhibition)	
	Human cells (WISH)	Mouse cells (L929)	Bovine cells (MDBK)	Cell growth	³ H-TdR incorporation
IFN- α 2 wild type	1.0×10^8	1.5×10^3	1.3×10^9	26	19
Mutant A (Phe48 $\rightarrow$ Ser)	1.9×10^8	2.8×10^3	5.6×10^8	21	18
Mutant B (Phe48 $\rightarrow$ Tyr)	1.4×10^8	2.1×10^3	2.1×10^9	24	17
Mutant C (Phe48 $\rightarrow$ Cys)	1.1×10^8	1.6×10^3	5.5×10^9	31	23
Mutant D (Gln49 $\rightarrow$ His)	1.2×10^8	1.8×10^3	1.1×10^9	22	22

Specific activities of the partially purified IFNs were calculated from the antiviral activities and the IFN contents of the preparations, both determined as described in Fig. 3 legend. The IFN concentrations required for 50% inhibition of Daudi cell growth or ³H-thymidine (TdR) incorporation were obtained from dose-response assays¹⁵. Cell numbers were determined in a Coulter counter model ZBI and ³H-TdR incorporation was measured as described by Hannigan *et al.*²⁴. Each value is the average of a duplicate determination.

murine IFN- α ₁, - α ₂ (ref. 16), murine IFN- β (ref. 17), bovine IFN- α ₁ to - α ₄ (D. Goeddel, personal communication) and bovine IFN- β ₁ to - β ₃ (ref. 18). In this case, the sequence of the mutated DNA, but not the composition of the protein, was verified. The specific antiviral activity of the purified mutated IFN determined on human (WISH) cells as above, was 1.3×10^8 U per mg, compared with 1.6×10^8 U per mg for wild-type IFN- α ₂.

If strict amino acid conservation at the positions examined is not essential for the major biological effects ascribed to IFN- α and - β , that is, antiviral and growth-inhibitory activity, what is the significance of the constancy of the amino acids at positions 48, 49 and 62 (and possibly at other sites)? There are several explanations. (1) Selection could have occurred on the basis of a criterion not assayed for in the present work. Thus, replacement of the conserved residues might impair an as yet unrecognized biological activity of IFN, or could result in a protein which is deleterious to the cell. (2) Mutations could lead to inadequate expression of the IFN gene by affecting transcription, translation or post-translational events such as secretion. (3) The structure of the IFN gene has evolved not only to optimize function, but to minimize the effect of point mutations on function, for example by generating a protein framework so stable that even a non-conservative amino acid change does not impair function. (4) The conservation of at least some amino acid residues could be due to chance and our findings a result of the small sample so far analysed. We have determined the number of changes between each human IFN species and an IFN consensus sequence reflecting the most commonly used amino acid in each position of the human α and β IFNs, and have estimated the number of positions that would be expected to coincide in all IFNs by Poisson analysis. By this admittedly naive approach, we estimate that about half the conserved positions in the human genes may have been retained by chance, indicating that our results for positions 48 and 49 could be ascribed to the small sample size. However, if the mutation at position 62 is taken into account, this possibility becomes considerably less likely, because this position is conserved in IFN- α and - β genes in three species.

A situation similar to the one we have generated artificially for the interferons may exist naturally in the case of the globins. For example, positions 127 and 141 of α -globin are conserved in the 48 mammalian species listed in the current protein sequence database of Dayhoff¹⁹. Nonetheless, variants of human haemoglobin containing replacements at these strictly conserved sites seem to be functionally unimpaired and the individuals bearing this polymorphic variation are healthy^{20,21}. In this case, explanations (2) and (4) seem less likely.

Thus, we conclude that the occurrence of conserved positions in related proteins, even when clustered, does not necessarily imply that these positions are essential for the function of the protein, or in other words: while all that is important is conserved, not all that is conserved need be important.

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- Weissmann, C. *et al. Phil. Trans. R. Soc. B* **299**, 7-28 (1982).
- Taniguchi, T. *et al. Nature* **285**, 547-549 (1980).
- Aguet, M. & Blanchard, B. *Virology* **115**, 249-261 (1981).
- Branca, A. A. & Baglioni, C. *Nature* **294**, 768-770 (1981).
- Joshi, A. R., Sarkar, F. H. & Gupta, S. L. *J. biol. Chem.* **257**, 13884-13887 (1982).
- Yonehara, S., Yonehara-Takahashi, M. & Ishii, A. *J. Virol.* **45**, 1168-1171 (1983).
- Alberts, B. *et al. in Molecular Biology of the Cell*, p. 215 (Garland, New York, 1983).
- Hutchison, C. A. III *et al. J. biol. Chem.* **253**, 6551-6560 (1978).
- Razin, A., Hirose, T., Itakura, K. & Riggs, A. D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4268-4270 (1978).
- Kössel, H., Buyer, R., Morioka, S. & Schott, H. *Nucleic Acids. Res. Spec. Publ.* **4**, s91-s94 (1978).
- Panayotatos, N., Fontaine, A. & Truong, K. *J. cell. Biochem. Suppl.* **7B**, 109 (1983).
- Dente, L., Cesareni, G. & Cortese, R. *Nucleic Acids Res.* **11**, 1645-1655 (1983).
- Taub, F. & Thompson, E. B. *Analyt. Biochem.* **126**, 222-230 (1982).
- Wallace, R. B. *et al. Nucleic Acids Res.* **9**, 879-894 (1981).
- Evinger, M. & Pestka, S. *Meth. Enzym.* **79**, 362-368 (1981).
- Shaw, G. D. *et al. Nucleic Acids Res.* **11**, 555-573 (1983).
- Higashi, Y. *et al. J. biol. Chem.* **258**, 9522-9529 (1983).
- Leung, D. W., Capon, D. J. & Goeddel, D. V. *Biotechnology* **2**, 458-464 (1984).
- Dayhoff, M. O. *Protein Sequence Database of the National Biochemical Research Foundation*, Sept. 1984 update (Washington, DC).
- Vella, F. *et al. Biochim. biophys. Acta* **365**, 318-322 (1974).
- Clegg, J. B., Weatherhall, D. J., Boon, W. H. & Mustafa, D. *Nature* **222**, 379-380 (1969).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).
- Moore, S. & Stein, W. H. *Meth. Enzym.* **6**, 819-831 (1963).
- Hannigan, G. E., Gewert, D. R., Fish, E. N., Read, S. E. & Williams, R. G. *Biochem. biophys. Res. Commun.* **110**, 537-544 (1981).
- Panayotatos, N. & Truong, K. *Nucleic Acids Res.* **9**, 5679-5688 (1981).
- Messing, J., Crea, R. & Seeburg, P. H. *Nucleic Acids Res.* **9**, 309-321 (1981).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning*, 170 (Cold Spring Harbor Laboratory, New York, 1982).
- Zoller, M. J. & Smith, M. *Nucleic Acids Res.* **10**, 6487-6500 (1982).
- Holmes, D. S. & Quigley, M. *Analyt. Biochem.* **114**, 193-197 (1981).
- Maxam, A. & Gilbert, W. *Meth. Enzym.* **65**, 499-560 (1980).
- Derynck, R. *et al. Nature* **287**, 193-197 (1980).
- Nagata, S. *et al. Nature* **284**, 316-320 (1980).

Yeast and mammalian *ras* proteins have conserved biochemical properties

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Mammalian *ras* oncogenes encode polypeptides of relative molecular mass (M_r) 21,000 (p21) which bind GTP and GDP^{1,2}. Oncogenic *ras*-encoded proteins differ from their normal homologues by an amino acid substitution for Gly 12, Ala 59 or

Gln 61 (refs 3–14). Recently, we and others have observed that normal p21, encoded by the *Ha-ras* gene, has a GTP hydrolytic activity that is reduced by the oncogenic substitutions Val 12 (refs 15–17) or Thr 59 (ref. 15). The yeast *Saccharomyces cerevisiae* contains two *ras*-related genes, *RAS^{sc1}* and *RAS^{sc2}* (refs 18, 19), the expression of either of which is sufficient for viability^{20,21}. *RAS^{sc1}* and *RAS^{sc2}* encode proteins of 309 (SC1) and 322 (SC2) residues which are 62% homologous to mammalian p21 in their 172-amino acid N-terminal sequences^{19,22}. We report here that the N-terminal domain of SC1 binds GTP and GDP and has a GTP hydrolytic activity that is reduced in the variants SC1[Thr 66] and SC1[Leu 68] which are analogous to oncogenic Ha[Thr 59] and Ha[Leu 61], respectively. These results suggest that yeast and mammalian *ras* proteins have similar biochemical and possibly biological functions.

To obtain expression of the SC1 protein, the 1.7-kilobase (kb) *Hind*III fragment containing *RAS^{sc1}* was subcloned into phage M13 mp9. By oligonucleotide-directed mutagenesis¹⁵, a *Hind*III site was introduced at a position analogous to that found at the 5' region of v-Ha-*ras*, creating *RAS^{sc1}* (*Hind*III). Mutations were also introduced to create variants *RAS^{sc1}*[Thr 66] and *RAS^{sc1}*[Leu 68]. Previously we reported the use of plasmid pRAS to obtain expression of v-Ha p21 and variants as 24,000-*M_r* proteins containing 20-amino acid N-terminal extensions^{15,23}. The *Hind*III fragment of pRAS, encoding amino acids 3–189 of v-Ha p21, was replaced by the 1,040-base pair (bp) *Hind*III fragment of *RAS^{sc1}* (*Hind*III) encoding SC1 residues 10–309, producing pSC1. Based on sequence homology, amino-acid position 10 of SC1 corresponds to position 3 of Ha p21. Plasmids pSC1[Thr 66] and pSC1[Leu 68] were then generated by replacing the 321-bp *Cl*AI/*Acc*I fragment of pSC1 with the corresponding fragments of *RAS^{sc1}*[Thr 66] and *RAS^{sc1}*[Leu 68]. For expression of only the N-terminal domains of the SC1 proteins, the above plasmids were digested with *Hinc*II followed by recirculation to remove the *Hinc*II fragment, producing pSC1N, pSC1N[Thr 66] and pSC1N[Leu 68]. In these plasmids, the codons for Leu 185 and Asp 186 are changed to that for a glycine residue and a nonsense codon, respectively.

Analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins produced by *Escherichia coli* JM101 cells transformed with pSC1[Thr 66] revealed small amounts of proteolytically labile novel polypeptides of *M_r* 30,000–40,000 (Fig. 1a, lanes 1, 2). Similar results were observed for cells transformed with pSC1 and pSC1[Leu 68] (not shown). In contrast, cells transformed with the plasmids pSC1N, pSC1N[Thr 66] or pSC1N[Leu 68] each expressed large quantities of a polypeptide of apparent *M_r* 23,000 (Fig. 1a, lane 3). All of the above polypeptides could be immunoprecipitated (data not shown) by the anti-v-Ha-*ras* monoclonal antibody YAG-259 (ref. 24).

The *ras*-related polypeptides expressed by *E. coli* cells transformed with pSC1[Thr 66] and pSC1N[Thr 66] possessed a GTP-specific autophosphorylating activity, as did Thr 59 mutants of Ha p21 (ref. 25). This activity, as well as GTP and GDP binding activities, resides in trypsin-resistant N-terminal domains that have the same apparent *M_r*, 22,000 (Fig. 1b). Peptide sequence analysis revealed that for Ha[Thr 59], the N-terminal trypsin cleavage site occurs between Arg 8 and Gly 9 of the fusion leader sequence, 12 amino acids preceding the authentic Ha p21 Met 1. We calculate from the size of this core protein and the sequence data for the proteolytic fragments that the trypsin-resistant core sequence extends to amino acid 169 of Ha p21. The trypsin resistance of the conserved N-terminal region of both yeast and mammalian *ras* proteins indicates that it may be an actual structural domain.

The stable N-terminal polypeptides expressed using plasmids pSC1N, pSC1N[Thr 66] and pSC1N[Leu 68] were purified to homogeneity as described in Fig. 2 legend. GTP hydrolytic activity co-purified with SC1N (Fig. 2a, top panel), as had been observed for normal Ha p21. Whereas GTP-specific autophosphorylating activity co-chromatographed with GDP binding activity for SC1N[Thr 66], a corresponding peak of GTP hydro-

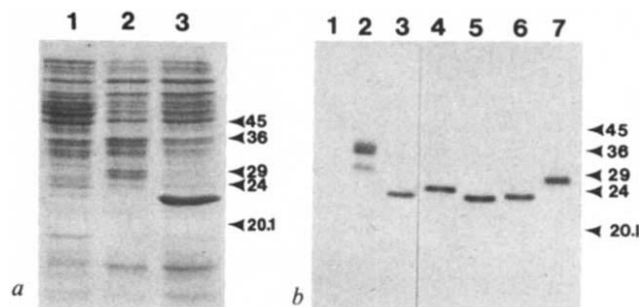


Fig. 1 a, Bacterial expression of yeast SC1 protein. Total cellular proteins were analysed by SDS-PAGE and visualized by staining with Coomassie brilliant blue. Each lane contained ~5 µg of protein. Lane 1, JM101; lane 2, JM101/pSC1[Thr 66]; lane 3, JM101/pSC1N. b, Trypsin treatment of SC1[Thr 66], SC1N[Thr 66] protein and Ha[Thr 59] p21. Crude lysates (lanes 1–3) and purified proteins (lanes 4–7) were incubated in autophosphorylation conditions with [γ -³²P]GTP (see Fig. 2 legend) for 1 h at 50 °C, with and without trypsin pre-treatment. Reactions were analysed by SDS-PAGE followed by autoradiography. Lane 1, JM101; lane 2, JM101/pSC1[Thr 66]; lane 3, trypsin-treated JM101/pSC1[Thr 66]; lane 4, SC1N[Thr 66]; lane 5, trypsin-treated SC1N[Thr 66]; lane 6, trypsin-treated Ha[Thr 59]; lane 7, Ha[Thr 59]. Lanes 1–3 contained ~12 µg protein each; lanes 4–7, ~1 µg each. The relative molecular masses ($\times 10^{-3}$) of reference proteins are shown.

Methods. Trypsin digestions were performed in buffer A (see Fig. 2 legend) without protease inhibitors and with 0.01 mg TLCK-treated trypsin (Sigma) per mg of protein for 1 h at 4 °C. Digestion was terminated by the addition of a threefold molar excess of soybean trypsin inhibitor (Sigma). SDS-PAGE²⁹ was performed with 12.5% polyacrylamide gels on a Bio-Rad Baby Gel apparatus at 30 mA constant current. Autoradiography was performed with Kodak XAR film.

Table 1 GTP hydrolysis by yeast and mammalian *ras*-encoded proteins

<i>ras</i> protein form	GTPase (mmol phosphate per min per mol protein · GTP)	Autophos- phorylation per min per mol protein · GTP
SC1N[Gly 19-Ala 66-Gln 68]	39.6 ± 9.4	0
SC1N[Gly 19-Thr 66-Gln 68]	<1.6 ± 0.2	1.0 ± 0.1
SC1N[Gly 19-Ala 66-Leu 68]	<0.3 ± 0.12	0
Ha[Gly 12-Ala 59-Gln 61]	17.7 ± 1.0	0
Ha[Gly 12-Thr 59-Gln 61]	2.0 ± 0.2	0.91 ± 0.22
Ha[Val 12-Ala 59-Gln 61]	1.4 ± 0.2	0
Ha[Arg 12-Ala 59-Gln 61]	<1.5 ± 0.6	0
Ha[Gly 12-Ala 59-Leu 61]	<0.36 ± 0.13	0

Yeast *ras*-encoded proteins were purified as described in Fig. 2 legend and Ha-*ras* proteins were purified as described previously¹⁵, with the addition of a second gel-filtration step over Sephadex G-150 for the [Leu 61] mutant. The preparations were assayed with 4 µM [γ -³²P]GTP for GTP binding, GTPase and autophosphorylating activities at 37 °C for 90 min. The results are expressed relative to the amount of [γ -³²P]GTP bound after 90 min (1.01–33.7 pmol). GTPase assay blank values were <20% of the measured activities for the normal yeast and Ha-*ras* proteins and 50–90% of the activities for the mutant variants. Autophosphorylation assay blank values were <1%. Data are the mean ± s.d. for six or seven determinations.

lytic activity was absent (Fig. 2a, bottom panel). As described below, the variant SC1N[Leu 68] also lacked GTPase activity. The same specific GTPase activity as measured for SC1N was observed (data not shown) using published methods¹⁵ in immune precipitates (monoclonal antibody YAG-259) of the bacterially expressed SC1 protein and of the native gene product from a yeast strain containing multiple copies of the *RAS^{sc1}* gene²⁶. Each of the purified SC1N variants demonstrated the same subtle but definite differences in electrophoretic mobility as the analogous Ha-*ras* mutants (Fig. 2b). The mutant SC1N[Thr 66] protein migrated more slowly than the wild-type SC1N protein, while the SC1N[Leu 68] protein migrated faster. An additional slower-migrating band representing the phos-

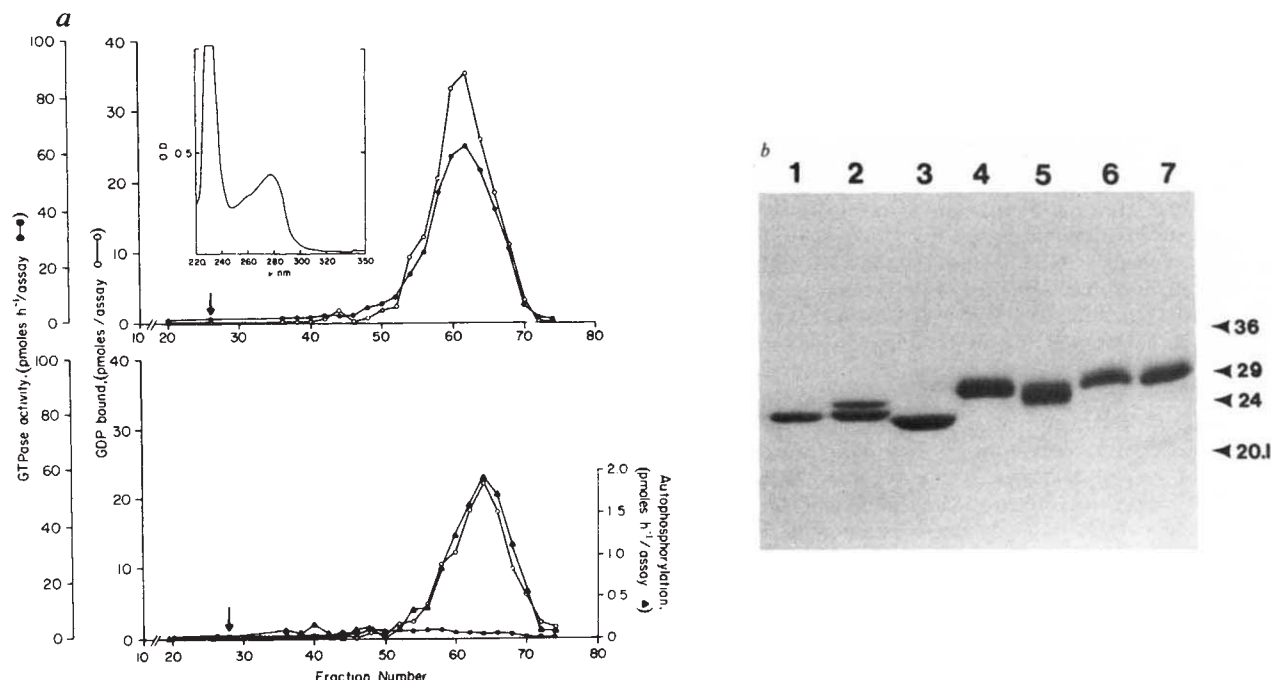


Fig. 2 *a*, Purification of SC1N proteins. Sephadex G-150 chromatography of SC1N (upper panel) and SC1N[Thr 66] (lower panel) proteins. Column fractions were diluted 1:10 with buffer A (see Methods) and assayed in parallel for ^3H -GDP binding ($\circ$), GTPase activity ($\bullet$) and autophosphorylation ($\blacktriangle$) at 37°C for 1 h. Void volumes for both chromatograms are marked by arrows. Inset: The UV spectrum of SC1N protein (0.25 mg ml^{-1}) was measured in buffer A with an IBM 1420 spectrophotometer using a 1-cm path-length cuvette. *b*, SDS-PAGE of purified bacterially expressed *ras*-encoded proteins. Lane 1, SC1N; lane 2, SC1N[Thr 66]; lane 3, SC1N[Leu 68]; lane 4, Ha; lane 5, Ha[Leu 61]; lane 6, Ha[Arg 12]; lane 7, Ha[Val 12]. Each lane contained $\sim 5\text{ }\mu\text{g}$ of protein except for lane 6, which contained $\sim 3\text{ }\mu\text{g}$. SDS-PAGE methods as described in the legend to Fig. 1.

Methods. Lysates were prepared as described previously¹⁵ with addition of 2 mM EGTA, 0.1% Trasylol (Calbiochem) and 50 μM phenylmethylsulphonyl fluoride (PMSF). Prepared supernatants were diluted 1:2 with buffer A (50 mM sodium HEPES pH 7.5, 1 mM sodium EDTA, 0.01% *n*-octylglucoside, 1 mM dithiothreitol, 2 mM EGTA, 0.1% Trasylol and 50 μM PMSF) and applied to a column ($1.5\times 20\text{ cm}$) of DEAE-Sephacel (Pharmacia) equilibrated with buffer A at 4°C . Elution of SC1N proteins with a 120 ml linear 0–1.0 M NaCl gradient was monitored by assays described below, SDS-PAGE and 260-nm and 280-nm absorbance. Pooled peak fractions ($<10\text{ ml}$) were applied to a column ($2.6\times 34\text{ cm}$) of Sephadex G-150 and 2-ml fractions were collected. Typically, 30–50 mg of SC1N protein, $>95\%$ pure by SDS-PAGE, were obtained. Nucleotide binding, autophosphorylation and GTPase activities were determined in solution by published methods¹⁵. Reactions were performed in 50 μl volumes of buffer B (50 mM sodium HEPES pH 8.0, 0.5 mM MgSO_4 , 100 mM NaCl, 1 mM dithiothreitol, 0.25 mM AMP-PNP) and 4 μM ^3H -GDP (binding) or 4 μM [γ - ^{32}P]GTP (autophosphorylation and GTPase). The GTPase assay reaction products were identified as GDP and orthophosphate by poly(ethyleneimine) cellulose chromatography^{2,15}. Protein concentration was determined with Bradford reagent³⁰ as supplied by Bio-Rad.

phorylated species was evident in preparations of purified SC1N[Thr 66] protein.

As has been observed for v-Ha p21 (ref. 27), SC1N proteins co-purify with bound guanine nucleotide. The endogenous nucleotide, identified by anion-exchange HPLC as GDP for SC1N, is present at $\sim 0.5\text{ mol per mol}$ protein and gives rise to the 260-nm shoulder on the 280-nm absorbance peak in the ultraviolet spectrum of SC1N (Fig. 2*a*, inset). The specificity of nucleotide binding was determined for SC1N protein by using unlabelled nucleotides as competitors in ^3H -GDP (2 μM) binding assays. Exactly as observed for Ha p21^{1,2}, GMP, cyclic GMP, ATP, AMP, adenylyl-5'-yl imidodiphosphate (AMP-PNP) and cyclic AMP did not compete, even at mM concentrations. GTP competed at μM levels while 1 mM ITP reduced binding of ^3H -GDP by 80%. To compare accurately the affinity of *ras* proteins for GTP relative to GDP, binding assays were performed with solutions containing equal amounts of both GDP and GTP, one of which was labelled with tritium. The affinities of SC1N and SC1N[Leu 68] for the two nucleotides were approximately equivalent except for a small preference for GTP that was more pronounced for SC1N[Leu 68] (data not shown).

To understand more fully the nature of the *ras* protein-mediated GTPase reaction, we measured in parallel the time courses for binding of [γ - ^{32}P]GTP and ^3H -GTP and the hydrolysis of [γ - ^{32}P]GTP by Ha, SC1N and SC1N[Leu 68] proteins (Fig. 3). The GTPase activity of SC1N protein was twice that of Ha p21, and the activities for both of these proteins were much greater than the GTPase activity of the SC1N[Leu 68]

protein. Nevertheless, ^3H -GTP binding proceeded at similar rates for the three proteins. The binding occurred in two stages with an initial burst followed by a slow increase. These kinetics are consistent with a proportion of the protein molecules having endogenous bound nucleotide. In contrast to ^3H -GTP binding, the binding of [γ - ^{32}P]GTP reached a constant value after 1 h (Fig. 3). The difference between the ^3H -GTP and [γ - ^{32}P]GTP binding curves reflects a steady state involving binding and hydrolysis.

To test further whether oncogenic mutations in general possess reduced GTP hydrolytic activities, we used previously described methods¹⁵ to prepare two new Ha-*ras* variants, one containing another substitution at position 12 (Ha[Arg 12]) and one a substitution at position 61 (Ha[Leu 61]). Figure 2*b* shows the SDS-PAGE analysis of the purified proteins, whereas Table 1 lists the rate constants for the conversion of [*ras* protein $\cdot$ GTP] to [*ras* protein $\cdot$ GDP] for the Ha p21 and SC1N variants. The GTPase activities of the different oncogenic mutants varied between <2 and 10% that of the normal *ras* proteins, possibly a reflection of the roles that the critical residues play in the GTPase mechanism; for example, some residues might be catalytic active-site residues and others might be involved in determining active conformations. Theoretical analyses have suggested that Gly 12 of p21 uniquely determines the conformation of the surrounding sequence^{10,28}. Perhaps the presence of four consecutive Gly residues in the SC1 sequence at positions analogous to 10–13 (Gly-Ala-Gly-Gly) of Ha p21 is responsible for SC1 having twice the GTPase activity of Ha p21.

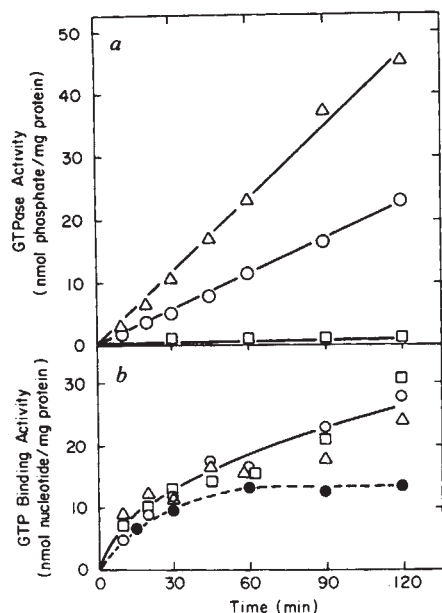


Fig. 3 Time course for GTPase and GTP binding activities of yeast and mammalian *ras*-encoded proteins. *a*, Assay mixtures containing 4 μ M [γ - 32 P]GTP were prewarmed to 37 °C and the reactions were initiated by addition of purified *ras* protein to final concentrations of 16.8 μ g ml $^{-1}$ (SC1N, Δ), 17 μ g ml $^{-1}$ (Ha, $\circ$) or 13.6 μ g ml $^{-1}$ (SC1N[Leu68], $\square$). At the indicated times, duplicate 50- μ l aliquots were removed, quickly chilled and analysed for 32 P-orthophosphate after charcoal treatment as described elsewhere¹⁵. *b*, Same as *a* except that assay mixtures contained 4 μ M 3 H-GTP (Δ , $\circ$, $\square$) or 4 μ M [γ - 32 P]GTP ($\bullet$, Ha) and were analysed for bound nucleotide by rapid filtration¹⁵. Protein was determined by the method of Bradford³⁰ as supplied by Bio-Rad.

It has been suggested that [p21 · GTP] is a growth-promoting complex which is deactivated on its conversion to [p21 · GDP]^{15–17}. Accordingly, the GTP hydrolytic activities reported here for yeast and mammalian *ras* proteins should correlate inversely with the relative effects of these proteins on biological function.

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1. Scolnick, E. M., Papageorge, A. G. & Shih, T. Y. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5355–5359 (1979).
2. Shih, T. Y., Papageorge, A. G., Stokes, P. E., Weeks, M. O. & Scolnick, E. M. *Nature* **287**, 686–691 (1980).
3. Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. *Nature* **300**, 148–152 (1982).
4. Tabin, C. J. *et al. Nature* **300**, 143–148 (1982).
5. Taparowsky, E. *et al. Nature* **300**, 762–765 (1982).
6. Capon, D. J. *et al. Nature* **304**, 501–507 (1982).
7. Yuasa, Y. *et al. Nature* **303**, 775–779 (1982).
8. Taparowsky, E., Shimizu, K., Goldfarb, M. & Wigler, M. *Cell* **34**, 581–586 (1983).
9. Sukumar, S., Notario, V., Martin-Zanca, D. & Barbacid, M. *Nature* **306**, 658–661 (1983).
10. Santos, E., Reddy, E. P., Pulciani, S., Feldmann, R. J. & Barbacid, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4679–4683 (1983).
11. Yuasa, Y. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 3670–3674 (1984).
12. Sekiya, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 4771–4775 (1984).
13. Dahr, R. *et al. Science* **217**, 934–937 (1982).
14. Tsuchida, N., Ryder, T. & Ohtsubo, E. *Science* **217**, 937–938 (1982).
15. Gibbs, J. B., Sigal, I. S., Poe, M. & Scolnick, E. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5704–5708 (1984).
16. McGrath, J. P., Capon, D. J., Goeddel, D. V. & Levinson, A. D. *Nature* **310**, 644–649 (1984).
17. Sweet, R. W. *et al. Nature* **311**, 273–275 (1984).
18. DeFeo-Jones, D., Scolnick, E. M., Koller, R. & Dahr, R. *Nature* **306**, 707–709 (1983).
19. Powers, S. *et al. Cell* **36**, 607–612 (1984).
20. Tatchell, K., Chaleff, D. T., DeFeo-Jones, D. & Scolnick, E. M. *Nature* **309**, 523–527 (1984).
21. Kataoka, T. *et al. Cell* **37**, 437–445 (1984).
22. Dahr, R., Nieto, A., Koller, R., DeFeo-Jones, D. & Scolnick, E. M. *Nucleic Acids Res.* **12**, 3611–3618 (1984).
23. Stein, R. B., Robinson, P. S. & Scolnick, E. M. *J. Virol.* **50**, 343–351 (1984).
24. Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. *J. Virol.* **43**, 294–304 (1982).
25. Gibbs, J. B., Ellis, R. W. & Scolnick, E. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2674–2678 (1984).
26. Temeles, G. L., DeFeo-Jones, D., Tatchell, K., Ellinger, M. S. & Scolnick, E. M. *Molec. cell. Biol.* **4**, 2298–2305 (1984).
27. Poe, M., Scolnick, E. M. & Stein, R. B. *J. biol. Chem.* (in the press).
28. Pincus, M. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 5253–5257 (1983).
29. Laemmli, U. K. *Nature* **227**, 680–684 (1970).
30. Bradford, M. *Analyt. Biochem.* **72**, 248–252 (1976).

Production of phenocopies by *Krüppel* antisense RNA injection into *Drosophila* embryos

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The demonstration that a specific messenger RNA can be functionally inactivated *in vivo* by hybridization to complementary polynucleotide sequences suggests a direct approach to the study of gene function in cells of higher organisms^{1,2}. The experiments described here were designed to inhibit, by complementary RNA sequences, a specific gene function affecting the fate of the *Drosophila* embryo. We used the SP6 vector *in vitro* transcription system³ to transcribe parts of the normally untranscribed (non-sense) strand of the *Krüppel* (*Kr*) gene into complementary *Kr* RNA (*Kr* antisense RNA). Wild-type *Drosophila* embryos, injected with this RNA, developed into phenocopies of *Kr* mutant embryos.

The *Kr* gene has an early zygotic function in controlling segmentation of the *Drosophila* embryo^{4–7}. Embryos homozygous for *Kr* mutations die before hatching and show a unique phenotype characterized by a deletion of adjacent thoracic and anterior abdominal segments^{4–7}. In amorphic alleles, all three thoracic and five anterior abdominal segments are deleted and replaced by a mirror image duplication of parts of the normal posterior abdomen (compare Fig. 1*a* and *b*) often including the dorsally located tracheal endings, the Filzkörper⁶. Some intermediate alleles have all thoracic and four abdominal segments deleted but no duplication (Fig. 1*c*) except that ectopic Filzkörper develop frequently close to the head region⁶. In weaker alleles, progressively fewer segments are deleted and the prothorax is always developed (Fig. 1*d*). The weakest detectable phenotype is observed in heterozygous *Kr* embryos showing small defects in the denticle bands of thoracic and anterior abdominal segments. Such embryos may hatch and survive to become adults⁶.

Another feature making the *Kr* gene useful for assessing sequence-specific inhibition of gene function is that the *Kr*⁺ gene is transcribed between the syncytial blastoderm stage and the beginning of germ-band extension, producing a rare 2.5-kilobase (kb) poly(A)⁺ RNA transcript⁷. Most of this transcript has been recovered in a 2.3-kb cDNA clone (U.B.R. *et al.*, manuscript in preparation). This cDNA was subcloned, in both orientations, into plasmid DNA containing the SP6 promoter³, *Kr* sense and antisense RNA were transcribed from these constructs with SP6 RNA polymerase and prepared for injection (see Fig. 2).

Wild-type embryos were injected at the syncytial blastoderm stage. Sense RNA had no specific effect on the embryonic phenotype, but embryos injected with *Kr* antisense RNA developed lethal *Kr* phenocopies in high frequency (Table 1*a* and Fig. 1*e, f*). Some of the phenocopies developed ectopic Filzkörper close to the head region ('strong *Kr* phenocopy'; Fig. 1*g*), as found in *Kr* mutants developing the intermediate phenotype⁶. This new potential of the thorax region to develop a structure from a dorsal-posterior position on the blastoderm fate map⁸ demonstrates unequivocally the production of *Kr* phenocopies in wild-type embryos. Extreme *Kr* phenocopies resembling the amorphic *Kr* phenotype (Fig. 1*b*), however, were not observed, indicating that *Kr*⁺ activity was not abolished completely.

To determine whether extreme *Kr* phenocopies could be produced in embryos with only half the normal *Kr*⁺ gene dose,

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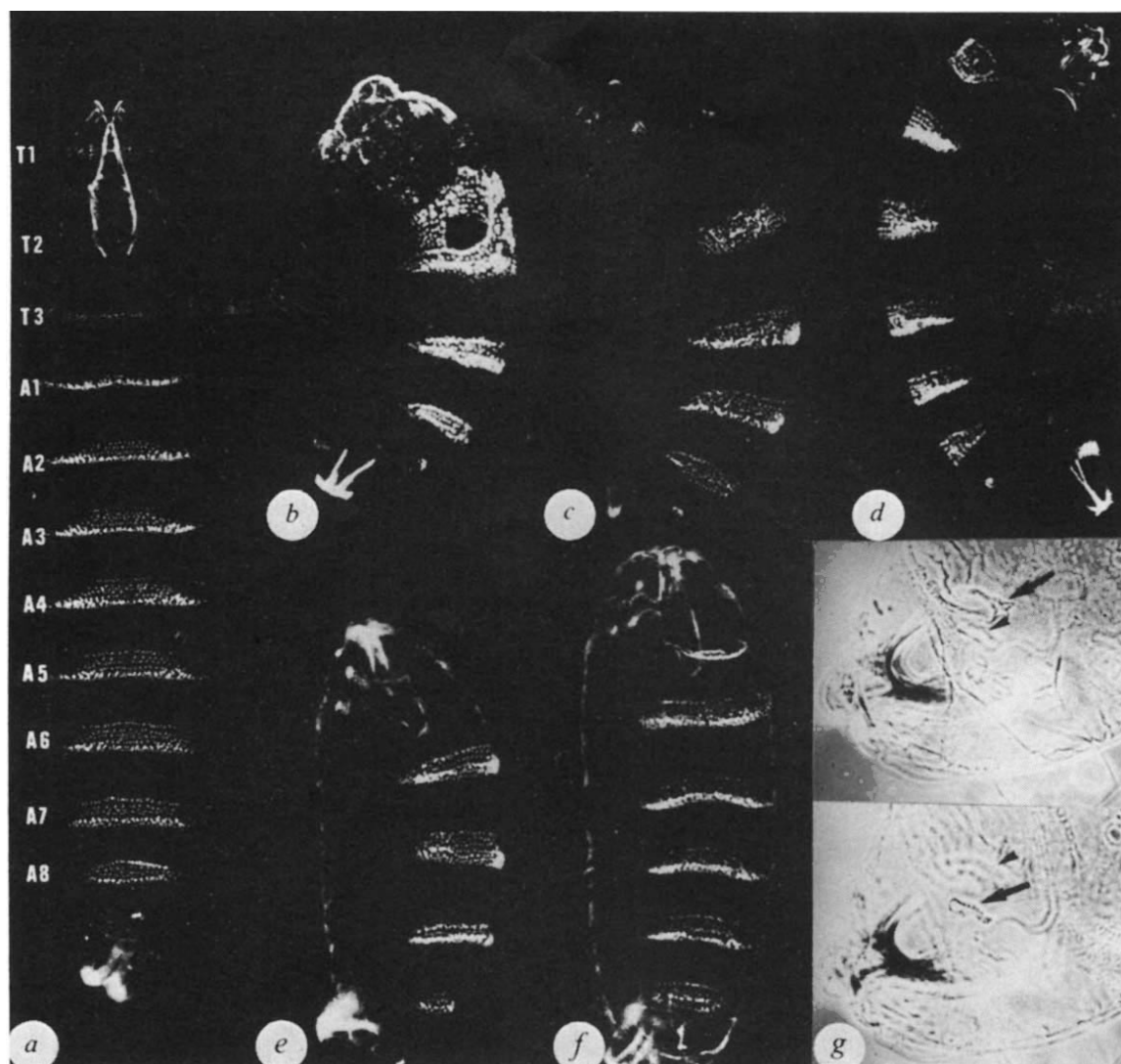
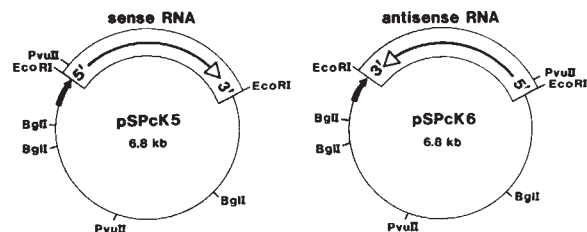


Fig. 1 Cuticle preparations showing the *Kr* phenotypes and *Kr* phenocopies produced by injecting embryos with *Kr* antisense RNA. *a*, Wild-type *Drosophila* larva after hatching. T1–3, pro-, meso and metathorax, respectively. A1–8, abdominal segments 1 to 8. Note the Filzkörper as a terminal posterior structure. *b*, Extreme *Kr* phenotype developed by amorphic *Kr*¹ homozygous embryos. Note the mirror image duplication of the sixth abdominal segment. *c*, Intermediate *Kr* phenotype^{6,7} frequently developing ectopic Filzkörper. Note the deletion of thoracic and four anterior abdominal segments. *d*, Weak *Kr* phenotype⁷. In these alleles, ectopic Filzkörper occur in low frequency or not at all. *e*–*g*, Examples of *Kr* phenocopies produced by wild-type embryos injected with *Kr* antisense RNA (4 µg µl⁻¹) as described in Table 1. *e*, Strong *Kr* phenocopy developed by 15% of the injected embryos listed in Table 1*a*. Examples of the ectopic Filzkörper material (out of focal plane) close to the head region are shown in the enlarged phase-contrast photographs (*g*). *f*, Weak *Kr* phenocopy developed by 22% of the embryos listed in Table 1*a*. Ectopic Filzkörper material was not observed in this class. *g*, Enlarged head region of a strong *Kr* phenocopy showing a pair of ectopic Filzkörper. Arrow, Filzkörper in focus; arrowhead, Filzkörper out of focal plane. Preparation of the embryos was as described in refs 6, 7. Details of the injection of the embryos and subsequent treatments are described in ref. 7 and in the legend to Table 1. The preparation and concentration of *Kr* antisense RNA is described in Fig. 2 legend.

we injected embryos of *Kr*¹/+ parents. To distinguish homozygous *Kr*¹ embryos from extreme *Kr* phenocopies produced in *Kr*¹/+ embryos, the *Kr*¹-bearing chromosome was marked with the recessive cuticle marker dopadecarboxylase (*Ddc*)^{6,7,9}. *Kr*¹ is a deletion mutation of the entire *Kr* region⁷. The *Kr*⁺*Ddc*⁺ chromosome was the multiply inverted balancer chromosome *SM1*¹⁰. As shown in Table 1*b*, *Kr* phenocopy production with *Kr*¹/*SM1* embryos was higher than with wild-type embryos, but again, no extreme *Kr* phenocopies were obtained. Phenocopy production was dose-dependent and *Kr* sense RNA produced no specific effect in the injected embryos (Table 1*b*). Note that injection *per se* sometimes results in nonspecific effects seen in the cuticular pattern of embryos. To distinguish these from embryos scored as weak *Kr* phenocopies, we used stringent criteria (see Table 1). Thus some weak *Kr* phenocopies could have been mistaken for injection artefacts but not vice versa, and our determination of weak *Kr* phenocopies is probably an underestimate.

In our most effective experiments, yielding ~50% of phenocopies (Table 1), we injected about 300 pl concentrated antisense RNA (4 µg µl⁻¹) having an average length of about 120 nucleotides (Fig. 2). These numbers correspond to about 5 × 10⁸ fragmented antisense RNA transcripts per injected embryo, which is of the order of 10³ times the number of *Kr*⁺ transcripts present in a wild-type embryo (U.B.R. *et al.*, manuscript in preparation). The weakest (4% phenocopies, Table 1*b*) required more than 50-fold excess, indicating that antisense RNA must be injected in excess over endogenous transcripts to result in a phenotypic response. These experiments did not elucidate the effective *in vivo* ratio of antisense RNA to endogenous transcript, as the stability of antisense RNA and the kinetic parameters of RNA-RNA duplex formation *in vivo* were not evaluated. We can, however, assume that most of the unprotected antisense RNA is degraded rapidly following injection. The mechanism by which antisense RNA inhibits *Kr*⁺ gene function is unknown. It may act in the nucleus by binding to the nascent *Kr* poly(A)⁺

Fig. 2 *In vitro* transcription templates for production of *Kr* sense and antisense RNA. A 2.3-kilobase (kb) *Kr* cDNA fragment was subcloned in the *EcoRI* site of pSP62-PL³. The orientation of the cDNA insert (open arrows) relative to the SP6 promoter sequence (closed arrow) is 3'-5' in pSPcK5 and 5'-3' in pSPcK6. SP6 RNA polymerase progresses clockwise (as shown) from the SP6 promoter. Truncated linear templates were prepared by digestion with the restriction enzymes *Bgl*I (pSPcK5) and *Pvu*II (pSPcK6) followed by extraction with phenol/chloroform/isoamyl alcohol (25/25/1) and water-saturated diethyl ether, followed by ethanol precipitation. Pellets were washed twice in 70% ethanol before redissolving to 1 $\mu\text{g } \mu\text{l}^{-1}$ in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. *In vitro* transcription reactions contained 6–7.5 $\mu\text{g } \mu\text{l}^{-1}$ linearized DNA, 40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 10 mM DTT, 4 mM spermidine, 25 units ml⁻¹ human placental ribonuclease inhibitor (Amersham), 500 μM each ribonucleoside triphosphate, 6–7 nM ³⁵S-UTP (800–1,200 Ci mmol⁻¹; NEN), and 100 units ml⁻¹ SP6-RNA polymerase (NEN). After incubation for 40 min at 37 °C, the amount of newly synthesized RNA was estimated by determination of the fraction of ³⁵S incorporated in trichloroacetic acid (TCA)-precipitable material. To estimate the percentage of RNA in full-length transcripts, parallel reactions producing RNA of high specific activity (1 $\times 10^8$ d.p.m. μg^{-1}) were subjected to electrophoresis in 5% acrylamide, 8 M urea gels (0.5 mm) run at 60 °C. Autoradiography showed that >80% of the newly synthesized RNA was full length. The transcription reaction was terminated by incubation at 65 °C for 5–10 min. Template DNA was removed by incubation at 37 °C for 10 min with 50 $\mu\text{g } \mu\text{l}^{-1}$ RNase-free DNase-I (BRL). After phenol extraction (see above), sodium acetate was added to 0.3 M, the RNA was precipitated with ethanol and the pellet was washed twice with 70% ethanol before redissolving in distilled water. For injections, the RNA was hydrolysed to a mean length of 120–130 bases by limited alkaline hydrolysis in carbonate buffer at 60 °C¹⁸. The lengths of the hydrolysis products were determined by electrophoresis and autoradiography as described above. After neutralization, the RNA was subjected to four rounds of solvent replacement with distilled water in a Centricon (Amicon) microconcentrator membrane device (exclusion limit 10,000 relative molecular mass). RNA recovered in the excluded volume was lyophilized in a centrifugal vacuum chamber and redissolved in 5 μl of injection buffer containing 0.1 mM sodium phosphate, pH 6.8, 5 mM KCl. Typically, 50–75% of the input mass of *in vitro* synthesized RNA was recovered, as shown by radioactivity measurements of TCA-precipitable material. Sense and antisense RNA strands were confirmed by an S₁-protection assay¹⁹ after hybridization to single-stranded cDNA with known orientation (U.B.R. *et al.*, in preparation).



RNA, leading either to its degradation or the prevention of its export to the cytoplasm. Alternatively, the antisense RNA may act in the cytoplasm by directly blocking the translation of *Kr*⁺ mRNA. There are precedents for the latter type of inhibition in prokaryotes: hybridization of short endogenous RNA transcripts to complementary regions of specific messenger RNA plays an important role in the normal translational regulation of some bacterial genes^{11–13}.

The potential of sequence-specific polynucleotide inhibition as a general method of genetic analysis at the cellular level has

been demonstrated recently^{1,2}. Our study on the *Kr* gene shows that excess injected antisense RNA efficiently inactivates a specific gene function, resulting in phenocopies equivalent to a mutant phenotype. As the *Kr* gene is transcribed into a rare transcript required at an early and brief period during embryogenesis⁷, this antisense RNA assay may affect the analysis of other early zygotic or maternal effect genes of *Drosophila*^{4,14–16}. This approach may not be a perfect paradigm for the analysis of many eukaryotic genes, particularly in organisms lacking well-developed genetic systems. It should be noted that our

Table 1 *Krüppel* phenocopy production resulting from injection of *Kr* sense and antisense RNA into *Drosophila* embryos

a, Injection into wild-type embryos									
Injected RNA	RNA concn ($\mu\text{g } \mu\text{l}^{-1}$)	No. of injected embryos	Developed embryos	Unpigmented (homozygous <i>Kr</i> ⁻¹) embryos¶	Pigmented (<i>Ddc</i> ⁺) embryos #	Hatched larvae* (%)	Injection artefacts† (%)	Weak <i>Kr</i> phenocopies‡ (%)	Strong <i>Kr</i> phenocopies§ (%)
Sense	3	300	263			193 (73)	70 (27)	0	0
Antisense	0.7	250	226			164 (72)	41 (18)	17 (7)	4 (2)
	4	207	174			71 (41)	38 (22)	38 (22)	27 (15)
b, Injection into embryos from <i>Ddc</i>ⁿ⁷ <i>Kr</i>¹/++ parents 									
Sense	0.6	300	229	60	169	141 (83)	28 (17)	0	0
	3	310	255	55	200	183 (92)	17 (8)	0	0
Antisense	0.15	250	218	39	179	138 (77)	34 (19)	5 (3)	2 (1)
	0.7	250	225	44	181	114 (63)	31 (17)	23 (13)	13 (7)
	4	398	248	59	189	21 (11)	75 (40)	39 (21)	54 (28)

Sense or antisense RNA was prepared as described in Fig. 2 legend from pSPcK5 and pSPcK6 templates, respectively, and injected into the middle of syncytial blastoderm stage embryos through the anterior or posterior pole. Data from anterior and posterior injection sites were pooled as no significant differences were associated with the site of injection. The injection volume was ~200–300 pl as described in ref. 7. RNA concentrations were determined as described in Fig. 2 legend and by A₂₆₀ measurements. Numbers in parentheses represent % of total (in a) or pigmented embryos (b) which develop cuticle.

* Excluding unfertilized eggs and embryos which died before cuticle formation.

† Embryos showing nonspecific defects in cuticular pattern. Note that this class probably includes some weak *Kr*-phenocopies showing additional nonspecific cuticular abnormalities.

‡ Embryos having deletions of mesothorax, metathorax, and at least one anterior abdominal segment but lacking ectopic Filzkörper. Embryos with cuticular wounds or other abnormalities (from injection) were classified as injection artefacts.

§ Embryos with segment deletions plus ectopic Filzkörper material in the deleted thoracic region. This class includes some embryos having injection artefacts, since these embryos possessed a positive criterion (ectopic Filzkörper) for scoring.

|| The ++ chromosome was the second chromosome balancer *SM1*¹⁰; thus the recessive larval cuticle marker *Ddc*ⁿ⁷ always cosegregates with *Kr*¹. Assuming normal segregation of chromosomes during gametogenesis, the expected ratio of embryonic genotypes was 1:2:1 for ++/++, *Ddc*ⁿ⁷ *Kr*¹/++, and *Ddc*ⁿ⁷ *Kr*¹/*Ddc*ⁿ⁷ *Kr*¹, respectively.

¶ Homozygous *Kr*¹ embryos were distinguishable from the embryos of the other two genotypic classes by their unpigmented cuticle resulting from the homozygous *Ddc*ⁿ⁷ mutation, and by the fully penetrant amorphic *Krüppel* phenotype.

Embryos possessing the *Ddc*ⁿ⁷ allele of *SM1* developed normally pigmented cuticle, and were thereby distinguishable from *Kr*¹ homozygotes. This category consists of embryos of the *Ddc*ⁿ⁷ *Kr*¹/++ and ++/++ genotypes in a 2:1 ratio, respectively. These comprise hatched larvae, embryos classified as injection artefacts, and weak and strong *Krüppel* phenocopies.

antisense RNA assay as a specific probe for endogenous gene expression is far from being optimized. For example, capping of antisense RNA to possibly prevent its immediate degradation of 'flipped gene' constructs transcribing from a strong and permanent or inducible promoter in transformed cells (see ref. 1) or transformed embryos¹⁷ have not yet been tried. The general usefulness of the antisense RNA assay may thus be evaluated as additional genes and other developmental systems are examined.

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1. Izant, J. G. & Weintraub, H. *Cell* **36**, 1007-1015 (1984).
2. Zamecnik, P. C. & Stephenson, M. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 280-284 (1978).
3. Green, M. R., Maniatis, T. & Melton, D. A. *Cell* **32**, 681-694 (1983).
4. Nüsslein-Volhard, C. & Wieschaus, E. *Nature* **287**, 795-801 (1980).
5. Gloor, H. *Arch. Jul. Klaus-Stiftung* **25**, 38-44 (1954).
6. Wieschaus, E., Nüsslein-Volhard, C. & Kluding, H. *Dev. Biol.* **104**, 172-186 (1984).
7. Preiss, A., Rosenberg, U., Kienlin, A., Seifert, E. & Jäckle, H. *Nature* **313**, 27-32 (1985).
8. Lohs-Schardin, M., Cremer, C. & Nüsslein-Volhard, C. *Dev. Biol.* **73**, 239-255 (1979).
9. Wright, T. R. F., Bewley, G. C. & Sherald, A. F. *Genetics* **84**, 287-311 (1976).
10. Lindsley, D. L. & Grell, E. H. *Carnegie Instn. Wash. Publ. No.* 627 (1968).
11. Mizuno, T., Chou, M.-Y. & Inouye, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1966-1970 (1984).
12. Simons, R. W. & Kleckner, N. *Cell* **34**, 683-691 (1983).
13. Coleman, J., Green, P. J. & Inouye, M. *Cell* **37**, 429-436 (1984).
14. Wieschaus, E., Nüsslein-Volhard, C. & Jürgens, G. *Wilhelm Roux Arch. dev. Biol.* **193**, 296-307 (1984).
15. Nüsslein-Volhard, C., Wieschaus, E. & Kluding, H. *Wilhelm Roux Arch. dev. Biol.* **193**, 267-282 (1984).
16. Jürgens, G., Kluding, H., Nüsslein-Volhard, C. & Wieschaus, E. *Wilhelm Roux Arch. dev. Biol.* **193**, 283-295 (1984).
17. Rubin, G. M. & Spradling, A. C. *Science* **218**, 348-353 (1982).
18. Cox, K. H., DeLeon, D. V., Angerer, L. M. & Angerer, R. C. *Dev. Biol.* **101**, 485-502 (1984).
19. Casey, J. & Davidson, N. *Nucleic Acids Res.* **4**, 1539-1552 (1977).

Glucocorticoid and progesterone receptors bind to the same sites in two hormonally regulated promoters

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The glucocorticoid receptor of rat liver recognizes nucleotide sequences near the promoter of mouse mammary tumour virus (MMTV) required for hormonal induction in gene transfer experiments¹⁻⁴. Similar nucleotide sequences have been found in the human metallothionein gene II_A (ref. 5) and in the chicken lysozyme gene⁶, the latter induced also by oestrogen, progesterone and androgens. In microinjection experiments, deletion of only 44 base pairs (bp) of the lysozyme promoter (from -208 to -164) results in coordinated loss of progesterone and glucocorticoid-dependent gene expression⁶. We show here that purified glucocorticoid receptor from rat liver and progesterone receptor from rabbit uterus yield similar or overlapping exonuclease III footprints in the promoter regions of MMTV and chicken lysozyme. Thus, the regulatory elements for different steroid hormones may be similar or at least share structural features.

In previous DNA binding experiments, we have used the partially purified rat liver glucocorticoid receptor⁷. To compare DNA binding properties of the receptors for two different steroid hormones, we chose the progesterone receptor of rabbit uterus⁸⁻¹⁰. The purification procedure involves an affinity chromatography on a deoxycorticosterone matrix in the presence of micromolar concentrations of dexamethasone, elution

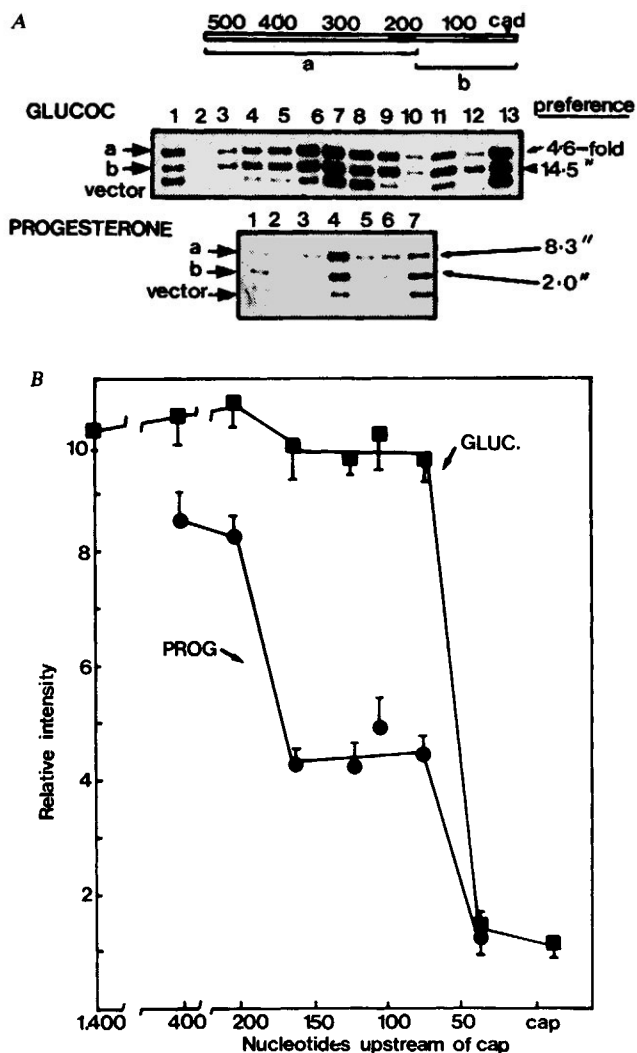
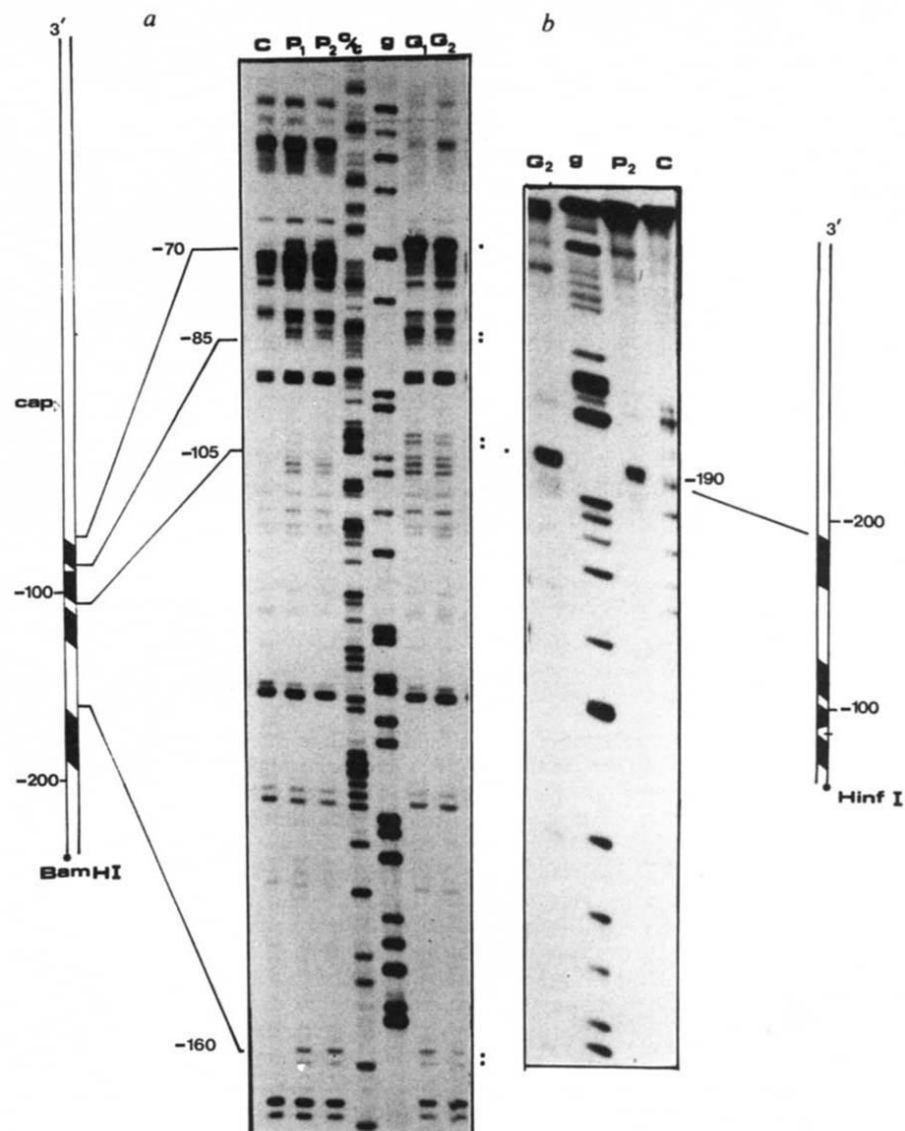


Fig. 1 DNA filter binding studies with the lysozyme promoter. **A**, Two plasmids, pUClys-550/-161 and pUClysΔ-164, containing the regions *a* and *b* of the lysozyme promoter (top diagram), were mixed in equimolar amounts with the vector pUC8, digested with *Eco*RI, and end-labelled with [α -³²P]dATP and the Klenow fragment of *Escherichia coli* DNA polymerase I (ref. 7). The labelled DNA (10 ng) was incubated with the partially purified glucocorticoid or progesterone receptors at 25 °C for 45 min and the protein-bound DNA fragments were analysed by filtration through nitrocellulose and electrophoresis on 1% agarose gels⁷. The autoradiograms were scanned with a microdensitometer and the intensity of each band relative to the input mixture was divided by the corresponding value in the input mixture to yield the extent of preferential binding, as shown in the right (maximal values). Glucocorticoid receptor: lanes 1 and 13, input DNA fragments; lane 2, fragments retained in the absence of the receptor; lanes 3-8, fragments bound in the presence of 5, 10, 20, 40, 60, 80 ng of receptor at 60 mM NaCl; lanes 9 and 10, fragments bound in the presence of 80 ng receptor at 100 and 150 mM NaCl; lanes 11 and 12, fragments bound by 80 ng receptor at 60 mM NaCl, in the presence of 25 and 50 ng calf thymus DNA. Progesterone receptor: lanes 1 and 7, input DNA fragments; lane 2, fragments retained in the absence of receptor; lanes 3 and 4, fragments bound by 5 and 10 ng of receptor at 60 mM NaCl; lane 5, fragments bound by 10 ng of receptor at 100 mM NaCl; lane 6, fragments bound by 10 ng of receptor at 60 mM NaCl in 25 ng calf thymus DNA. **B**, Summary of filter binding data with deletion mutants. A series of plasmids containing progressive 5'-deletions in the lysozyme promoter region was used for filter binding experiments with the glucocorticoid receptor (■) or the progesterone receptor (●) similar to those shown in **A**. The end-points of the deletions are shown in the abscissa. The ordinate gives the average and when indicated the standard error for the relative intensity of the corresponding bands in the autoradiograms, calculated as described in **A**.

Fig. 2 Exonuclease III footprints of the MMTV promoter. The indicated restriction fragments were labelled at the 5' ends (*) with [γ - 32 P]ATP and T4 polynucleotide kinase and subjected to exonuclease III digestion (37 °C, 5 min, 30 units enzyme in 60 μ l or 100 μ l assay at 5 mM MgCl₂) without (C) or with previous incubations with glucocorticoid receptor (G₁, 160 ng; G₂, 250 ng) or progesterone receptor (P₁, 80 ng; P₂, 120 ng). Aliquots of the end-labelled fragments were also subjected to guanine (g) and pyrimidine (c/t) base-specific chemical sequence analysis¹⁴. After digestion the samples were phenol extracted and electrophoresed in 6% acrylamide-urea sequencing gels¹⁴. The numbers refer to positions upstream of the initiation of transcription 'cap'.



with Org 2058 (a synthetic progestin which does not bind to glucocorticoid receptor)⁸ and differential chromatography on phosphocellulose (S.J. and M.B., in preparation). The final preparation is about 30% pure and exhibits two main protein bands of relative molecular mass (M_r) 81,000 and 105,000 which can be covalently labelled with steroid by irradiation¹⁰. This preparation is free of any contaminating glucocorticoid receptor as determined by binding of 3 H-triamcinolone acetonide or with monoclonal antibodies against the glucocorticoid receptor¹¹.

We have detected previously two binding sites for the glucocorticoid receptor in the promoter region of the chicken lysozyme gene, a strong site at around position -60 containing the hexanucleotide 5'-TGTTCT-3' in the lower strand and a weak site at around -180 (ref. 6). The latter binding site overlaps the region responsible for hormone-dependent expression of the lysozyme promoter in microinjection experiments⁶. We here compare the relative affinities of these two sites for the partially purified progesterone receptor from rabbit uterus. A restricted plasmid containing the binding site at -60 is bound preferentially by the glucocorticoid receptor but only weakly by the progesterone receptor (Fig. 1A). A restricted plasmid containing the binding site at -180, however, showed a higher affinity for the progesterone than for the glucocorticoid receptor. Figure 1B shows a summary of results obtained with a series of 5' deletion mutants using the nitrocellulose filter binding assay. With the glucocorticoid receptor, only the strong binding site

between -39 and -74 is detected, because DNAs containing this site are quantitatively retained on the filters. With the progesterone receptor, two binding sites are observed clearly, one between -164 and -208 and another between -39 and -74; the affinity for the latter site is lower than with the glucocorticoid receptor.

A more precise analysis of the nucleotide sequences recognized by each hormone receptor requires footprint experiments. As the concentration of progesterone receptor in partially purified form was not sufficient to obtain quantitative binding of DNA fragments required for DNase I protection experiments, we decided to use the very sensitive exonuclease III footprint assay¹². In this assay a signal is observed when the progression of the exonuclease from the 3' end towards the 5'-labelled end is blocked by the presence of a bound protein. To test the validity of this method for the localization of DNA-bound receptors, we performed experiments with the MMTV promoter where defined DNase I footprints have been established².

Our results (Fig. 2) show that in addition to the half-size signals there are several stops in the absence of receptor, probably representing sequence-dependent pauses in the exonuclease digestion^{12,13}. Only those stops absent from the controls are due to receptor binding. The limits of the exonuclease III footprints originated by glucocorticoid receptor binding coincide with the limits of the previously described DNase I footprints² (Fig. 2a). In Fig. 2b, only the stop at -190 is shown, as this strong binding

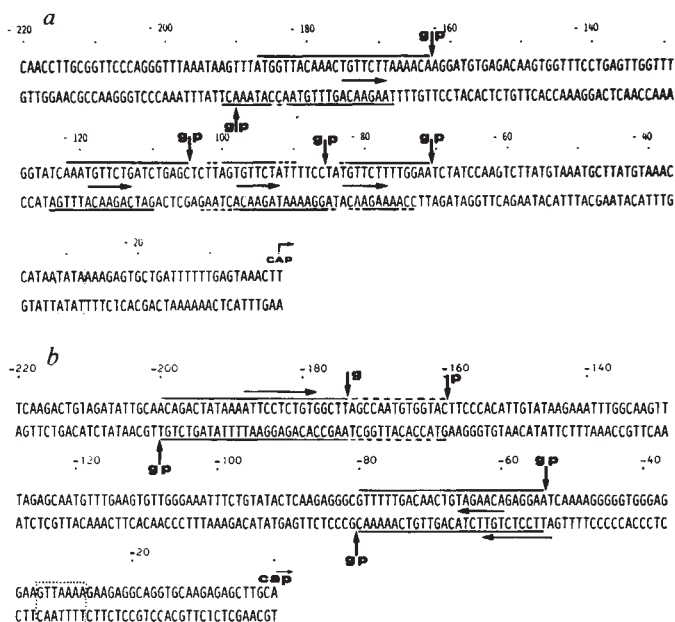


Fig. 3 Nucleotide sequences around the promoters of *a*, MMTV and *b*, chicken lysozyme gene. The DNase I footprints obtained with glucocorticoid receptor in the MMTV promoter are underlined². In the lysozyme promoter region the underlined sequences indicate the exonuclease III footprints. The hexanucleotide 5'-TGTCT-3' is indicated by horizontal arrows between the strands. Also shown by horizontal arrows in the lysozyme promoter is the decanucleotide 5'-ATTCCTCTGT-3' present in both binding sites⁶. The limits of the exonuclease footprints are indicated by vertical arrows labelled, g for glucocorticoid receptor; p for progesterone receptor, or gp for both. The TATA boxes and the 'cap' sites are indicated. Numbers refer to positions upstream of the initiation of transcription.

site is almost quantitatively occupied by the receptor, thus preventing the exonuclease III molecules from progressing towards the weaker sites further downstream. Therefore, we do not know whether the limits of the footprints in the lower strand are the same for both hormone receptors.

A comparison of the exonuclease III stop signals obtained with the rabbit progesterone receptor and with the rat glucocorticoid receptor shows that all five stops are located at the same positions but exhibit different intensities (Fig. 2). The stops at positions -70, -85 and -105 are much weaker with the progesterone receptor, whereas the signal at position -160 is weaker with glucocorticoid receptor, probably due to the higher frequency of occupancy of the other sites. Thus in the promoter region of MMTV, the progesterone receptors of rabbit uterus bind to the same sites as the glucocorticoid receptors of rat liver, although the relative affinities of each receptor for the individual sites are different (Fig. 3a).

In the promoter region of the chicken lysozyme gene we find two binding sites for each receptor. Three of the four exonuclease III stops detected are at the same positions (at -200, -80 and -54) with both glucocorticoid and progesterone receptor (Fig. 4). Although the stops at positions -54 and -80 are stronger with the glucocorticoid receptor, the stop at -200 is relatively stronger with the progesterone receptor.

The 3' limit of the binding site at -180 differs for both receptors. The glucocorticoid receptor yields a signal at position -174, whereas in the presence of the progesterone receptor no signal is observed in this position (Fig. 4). Instead the progesterone receptor causes a clear stop at position -160, where no signal is observed with the glucocorticoid receptor. Thus, the length of exonuclease III footprints is 26 bp for both receptors in the binding site around -60, whereas in the binding site at -180, the glucocorticoid receptor still occupies 26 bp, but the progesterone receptor protects 40 bp (Fig. 3b).

Together with the results of the MMTV experiments, these

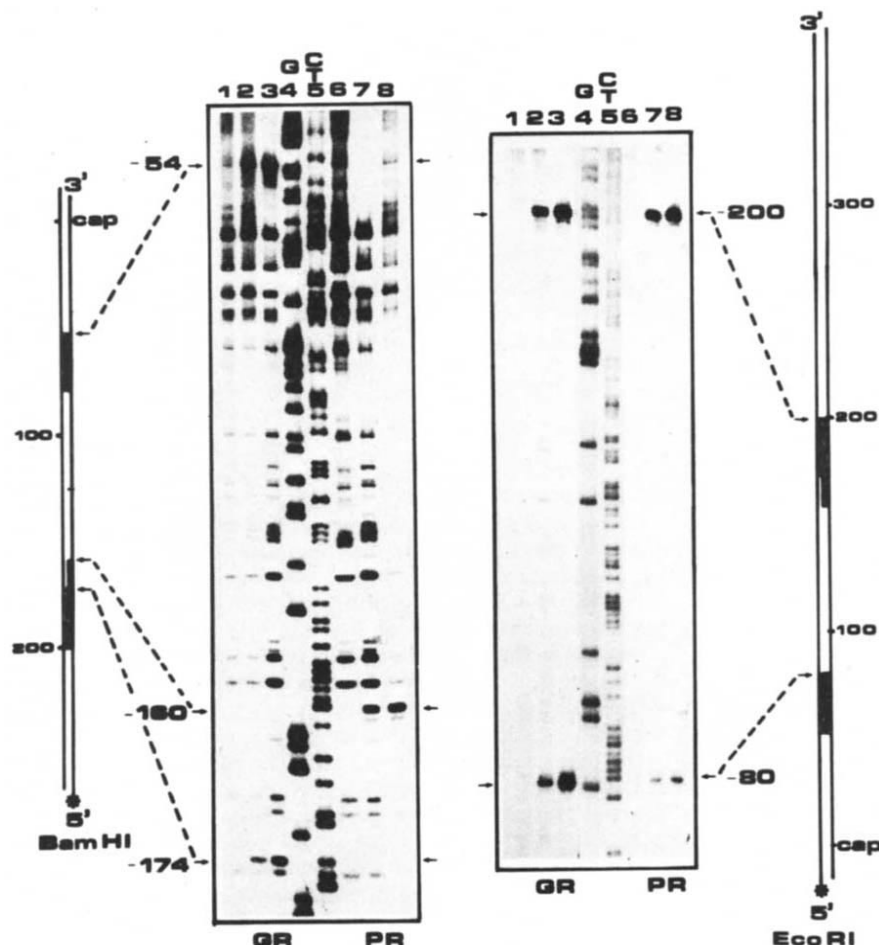


Fig. 4 Exonuclease footprint of the lysozyme promoter. The indicated restriction fragments, labelled at the 5' end, were subjected to exonuclease footprint and base-specific sequencing reactions as described in the legend to Fig. 2. Lanes 1 and 6, control reactions without added receptors; lanes 2 and 3, exonuclease footprints in 100 and 200 ng of glucocorticoid receptor (GR), respectively; lanes 7 and 8, exonuclease footprints in equivalent amounts of progesterone receptor (PR); lanes 4 and 5, G and C/T sequencing reactions, respectively¹⁴. The stops in the upper strand are shown in the left panel; those in the lower strand in the right panel.

results allow the following conclusions: (1) The progesterone receptor of rabbit uterus can bind to the same sites as the glucocorticoid receptor of rat liver; (2) when the hexanucleotide 5'-TGTTCT-3' is part of the binding site, the limits of the exonuclease III footprints are the same for both receptors; (3) the differences between glucocorticoid and progesterone receptors in terms of relative affinity to different sites suggest that the recognition mechanism is not identical for both receptors; (4) in the binding site at -180 of the lysozyme promoter, the different length of the footprints observed with both receptors confirms that the sequences recognized by the progesterone receptor are not necessarily identical to those bound by glucocorticoid receptor.

The functional significance of the interaction between the progesterone receptor and the MMTV promoter remains obscure as we have not found any suggestion of a progesterone effect on MMTV expression. For the chicken lysozyme gene, there is a good correlation between the sequences responsible for glucocorticoid and progesterone-dependent gene expression⁶. As this gene, as well as the other egg white protein genes, are induced by four classes of steroid hormones, oestrogen, progestins, glucocorticoids and androgens acting through their own receptors¹⁵, we wondered about the general validity of our findings. A revision of the literature shows that the ovalbumin promoter contains the sequence 5'-GGGTAACAATGTGTTT-3' at position -177 in the lower strand in a region thought to bind the progesterone receptor and to be required for hormonal regulation¹⁶. This sequence is 88% homologous to the consensus sequence for the binding site of the glucocorticoid receptor⁵. In the region of the chicken vitellogenin promoter that is protected against DNase I by the oestrogen receptor¹⁷, we find the sequence 5'-CGGCATCAATGTGTTCT-3' at position -587 in the lower strand; that is, over 90% homologous to the above-mentioned consensus⁵. Similar sequences are found in the promoter regions of the genes for an androgen-dependent rat prostatic protein¹⁸ and for an ecdysone-inducible *Drosophila* gene¹⁹. Preliminary experiments show that some of these elements actually bind the rat liver glucocorticoid receptor (C.S., unpublished). Thus, it seems that the regulatory elements for different steroid hormones, not only progesterone and glucocorticoids, are either similar or at least share structural features. A definitive solution of this question will come from an analysis of the contact sites between the different receptors and the DNA binding sites, similar to that performed with the MMTV promoter and the glucocorticoid receptor²⁰. Finally, it will be useful to have a test for distinguishing between productive and non-productive receptor-DNA interactions. Receptor binding to DNA probably is only the first step in a chain of events; subtle differences in the quality of the interaction may determine its functional consequences.

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Metallothionein gene cluster is split by chromosome 16 rearrangements in myelomonocytic leukaemia

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The metallothioneins (MTs) are a family of proteins of low relative molecular mass which bind heavy-metal ions¹. MTs exist in several molecular forms (MT-I, MT-II) and are encoded by a multi-gene family containing at least 14 closely related genes and pseudogenes^{1,2}. These proteins function in the regulation of trace-metal metabolism, the storage of these ions in the liver, and as a protective mechanism against heavy-metal toxicity³. Somatic cell hybridization has shown that most MT genes, including the functional MT genes (*MT1A*, *MT1B*, *MT2A*), lie on human chromosome 16 (refs 4, 5). Using *in situ* hybridization^{6,7}, we have now localized the MT genes to band q22 of chromosome 16. This chromosomal band is also a breakpoint in two specific rearrangements, the *inv*(16)(p13q22) (ref. 8) and *t*(16;16)(p13;q22) rearrangements, found in a subgroup of patients with acute myelomonocytic leukaemia (AMML). Hybridization of a MT probe to malignant cells from two patients with an *inv*(16) showed labelled sites on both arms of the inverted chromosome, indicating that the breakpoint at 16q22 splits the MT gene cluster. Similar results were obtained when this probe was hybridized to metaphase cells from two patients with a *t*(16;16). These results suggest that the MT genes or their regulatory regions⁹ may function as an 'activating' sequence for an as yet unidentified cellular gene located at 16p13.

To sub-localize the MT gene cluster on chromosome 16, we performed *in situ* hybridizations with a complementary DNA probe of the *MT2A* human gene (*hMT-II_A*)¹⁰ to normal human metaphase chromosomes. Metaphase cells obtained from phytohaemagglutinin-stimulated peripheral blood lymphocytes were hybridized with a radiolabelled probe using methods modified from those of Harper and Saunders^{6,7}. Of 100 metaphase cells examined, 25 were labelled on region 2 (bands q21-q24) of the long arm of one or both chromosomes 16 (Fig. 1). These sites represented 10.7% (29/271) of all labelled sites ($P < 0.005$); the largest cluster of grains was observed at 16q22.

In addition to the localization on chromosome 16, we observed a significant cluster of grains on 1p34-p36 (15/271 sites, 5.5%, $P < 0.005$), thereby sub-localizing another MT gene. These data agree with those of Schmidt *et al.*, who determined that the remaining MT genes are dispersed to at least four other autosomal sites on chromosomes 1, 4, 18 and 20 (ref. 4). These workers localized two *EcoRI* fragments (6.2- and 2.8-kilobase (kb) bands) of genomic DNA that hybridized with MT cDNA to chromosome 1. The 6.2-kb band was mapped to the distal two-thirds of the short arm of chromosome 1 and it was suggested that the smaller fragment was on the long arm of this chromosome. However, these genes are not expressed in human-mouse cell hybrids, and are likely to be transcriptionally silent⁵.

The localization of the functional MT genes to 16q22 is particularly interesting in view of the recent identification of a nonrandom chromosomal abnormality in a human tumour involving this chromosomal region. An inversion of chromosome 16 [*inv*(16)(p13q22)] is present in 25% of patients with AMML and is associated with unique morphological and cytochemical abnormalities of bone marrow eosinophils⁸. Two less common abnormalities also showing these features are a deletion of chromosome 16 [*del*(16)(q22)]¹¹ and a reciprocal translocation involving the two homologues of the chromosome [*t*(16;16)

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1. Payvar, F. *et al.* *Cell* **35**, 381-392 (1983).
2. Scheidereit, C., Geisse, S., Westphal, H. M. & Beato, M. *Nature* **304**, 749-752 (1983).
3. Chandler, V. L., Maler, B. A. & Yamamoto, K. R. *Cell* **33**, 489-499 (1983).
4. Hynes, N. H. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **80**, 3637-3641 (1983).
5. Karin, M. *et al.* *Nature* **308**, 513-519 (1984).
6. Renkawitz, R., Schütz, G., von der Ahe, D. & Beato, M. *Cell* **37**, 503-510 (1984).
7. Geisse, S. *et al.* *EMBO J.* **1**, 1613-1619 (1982).
8. Fleischmann, G. & Beato, M. *Biochim. biophys. Acta* **540**, 500-517 (1978).
9. Fleischmann, G. & Beato, M. *Molec. cell. Endocr.* **16**, 181-197 (1979).
10. Westphal, H. M., Fleischmann, G. & Beato, M. *Eur. J. Biochem.* **119**, 101-106 (1981).
11. Westphal, H. M., Moldenhauer, G. & Beato, M. *EMBO J.* **1**, 1467-1471 (1982).
12. Shalloway, D., Kleinberger, T. & Livingston, D. M. *Cell* **20**, 411-422 (1980).
13. Vocke, C. & Bastia, D. *Cell* **35**, 495-502 (1983).
14. Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499-560 (1980).
15. Moen, R. C. & Palmiter, R. D. *Dev. Biol.* **78**, 450-463 (1980).
16. Dean, D., Knoll, B. J., Riser, M. E. & O'Malley, B. W. *Nature* **305**, 551-554 (1983).
17. Jost, J. P., Seldran, M. & Geiser, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 429-433 (1984).
18. Parker, M., Hurst, H. & Page, M. J. *Steroid Biochem.* **20**, 67-71 (1984).
19. Möritz, Th., Edström, J. E. & Pongs, O. *EMBO J.* **3**, 289-295 (1984).
20. Scheidereit, C. & Beato, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3029-3033 (1984).

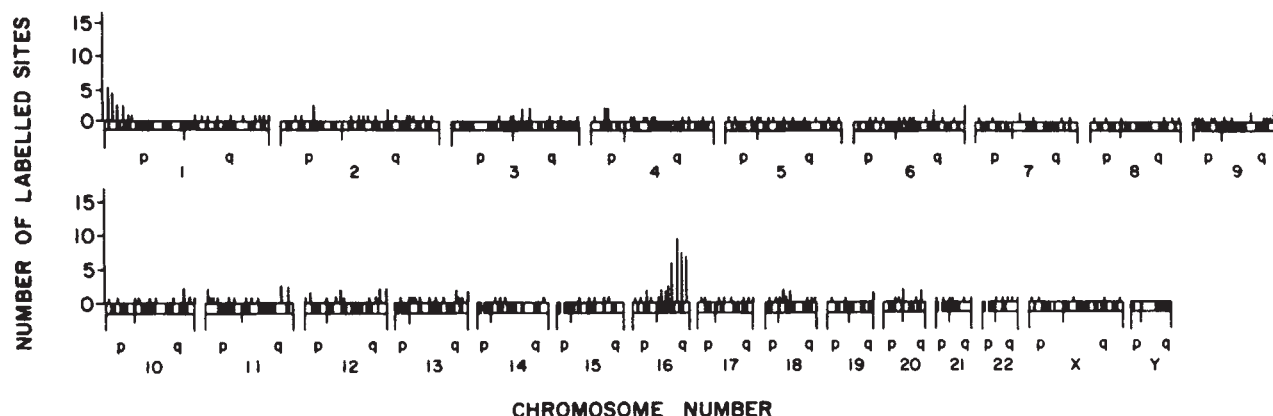


Fig. 1 Distribution of labelled sites in 100 normal metaphase cells. The major cluster of grains is on region q2 of chromosome 16. A second cluster of grains is on region p3 of chromosome 1. Human metaphase cells prepared from phytohaemagglutinin-stimulated peripheral blood lymphocytes were hybridized with the ^3H -labelled pH-MT-II₃ plasmid DNA containing the full coding region plus the untranslated 5'- and 3'-flanking regions of human *MT2A* cDNA¹². The *MT2A* coding region cross-hybridizes to all known *MT* genes⁶. Radiolabelled probes were prepared by nick-translation of the entire plasmid with all four ^3H -labelled deoxynucleotide triphosphates to specific activity 3.5×10^7 d.p.m. per μg . *In situ* hybridization was performed as described previously⁷.

(p13;q22)]¹². As in the case of the *inv*(16), the breakpoint in the long arm is located at band q22. A complex translocation involving both the short and long arms of one chromosome 16 and chromosome 1 [t(1;16)(q32;p13;q22)] has also been described¹³. Of the two junctions that result from the *inv*(16) and t(16;16) rearrangements, namely proximal 16p13-distal 16q22 and proximal 16q22-distal 16p13 (Fig. 2), only the latter junction is conserved. The former junction has been replaced by a reciprocal exchange of material with chromosome 1, suggesting that the conserved junction created by the movement of distal 16p13 to proximal q22 is the critical gene rearrangement.

To determine whether the *MT* gene cluster is altered by these rearrangements, we hybridized the *MT2A* cDNA probe to metaphase chromosomes from leukaemic bone marrow cells from two patients with an *inv*(16) rearrangement (Table 1). Of 111 metaphase cells examined from patient 1, 33 (29.7%) were labelled on either the normal chromosome 16 or the *inv*(16), or on both. Both chromosomes 16 were specifically labelled ($P < 0.005$). Analysis of the distribution of grains from 23 labelled normal homologues and 25 labelled inverted chromosomes 16 (Fig. 2) revealed that 14 labelled sites (3.9% (14/355) of all labelled sites) were on the normal chromosome 16 at bands q21-q24. In the inverted chromosome, 12 labelled sites (3.4%) were noted on the short arm (8 grains were located at the junction of 16p13 and q22) and 13 labelled sites (3.7%) were observed on the long arm (6 grains were identified at the junction of 16q22 and 16p13). Analysis of cells from a second patient showing this rearrangement gave similar results (Table 1). Thus, this chromosomal rearrangement splits the *MT* gene cluster and relocates a portion of this gene cluster to a new site at 16p13.

As in the *inv*(16) rearrangement, the t(16;16) involves an exchange of material at the same breakpoints between the short

and long arms of the chromosome 16 homologues, with the result that one has additional material on the short arm (16p+) and one lacks material from the long arm (16q-). *In situ* hybridization of the *MT2A* probe to metaphase cells from two patients with a (16;16) translocation revealed that the *MT* gene cluster is also divided by this rearrangement, and a portion of this cluster is relocated from the 16q- chromosome to 16p of the 16p+ chromosome (Table 1). Figure 3 shows the distribution of grains from 27 labelled chromosomes 16 from patient 3. Of 75 metaphase cells, 20 (27%) showed labelling of one or both chromosomes 16; both homologues were significantly labelled ($P < 0.005$). The 16p+ chromosome (Fig. 3, left) had 8 labelled sites (8/184, 4.3%) over 16q22-q24, as well as 4 grains in the short arm, at the 16p13-16q22 breakpoint junction. Four labelled sites were noted at the breakpoint junction on the long arm of the 16q- (Fig. 3, right). Similar results were obtained for a second patient with the (16;16) translocation (patient 4, Table 1).

There are several mechanisms by which *MT* genes may be integrally involved in the pathogenesis of leukaemia affecting the myeloid series. Mature eosinophils and granulocytes have a high zinc content in their granules, and both monocytes and granulocytes store and secrete several zinc metalloenzymes¹⁴. *MT* functions in the intracellular binding and storage of zinc and may therefore have an important role in granulocyte and monocyte differentiation. It is noteworthy that the specific morphological abnormality associated with this leukaemia is the accumulation in the bone marrow of eosinophils containing immature and dysplastic granules⁸. Other metal-ion-binding and transporting proteins, for example, transferrin and the transferrin receptor, also seem to have an integral role in the control of proliferation of certain cells¹⁵.

Table 1 Analysis of *in situ* hybridizations of malignant cells from patients with acute myelomonocytic leukaemia

Patient no.	Chromosomal abnormality	No. of metaphase cells analysed	No. of labelled sites	No. of cells labelled on chr. 16 (%)	No. of labelled sites (%)				
					Normal chr. 16	<i>inv</i> (16)		t(16;16) 16p+	
						p arm	q arm	q arm	p arm
1	<i>inv</i> (16)	111	355	33 (30%)	14 (4%)	12 (3.4%)	13 (3.7%)	—	—
2	<i>inv</i> (16)	50	101	88 (36%)	7 (7%)	6 (6%)	4 (4%)	—	—
3	t(16;16)	75	184	20 (27%)	—	—	—	8 (4.3%)	4 (2.2%)
4	t(16;16)	22	62	8 (36%)	—	—	—	3 (5%)	5 (8%)
									16q-q arm
									4 (2.2%)
									3 (5%)

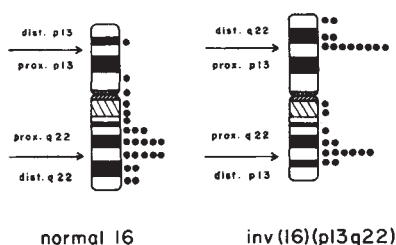


Fig. 2 Distribution of labelled sites on 23 normal (left) and 25 rearranged (right) chromosomes 16 from 111 metaphase cells from an AMML patient with an *inv(16)* rearrangement. The arrows identify the breakpoints on the normal chromosome 16 and the breakpoint junctions observed in the *inv(16)(p13q22)*.

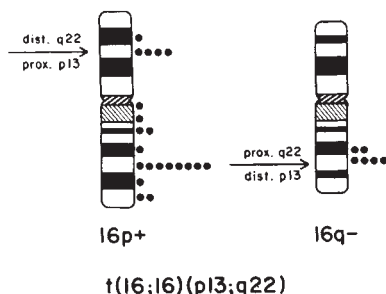


Fig. 3 Distribution of labelled sites on chromosome 16 homologues from 75 metaphase cells from an AMML patient with a *t(16;16)(p13;q22)* rearrangement. The 16p+ and 16q- chromosomes are on the left and right, respectively; the arrows identify the breakpoints.

Alternatively, because the normal function of more than one gene may be altered by a chromosomal rearrangement, it is possible that the transcriptional control elements of *MT* are involved in the activation of an as yet unidentified cellular gene, located on 16p13, whose function is analogous to that of a cellular oncogene. In this regard, it will be important to identify the precise *MT* gene involved in this rearrangement, as there are several known differences in the control elements associated with the *MT1A* and *MT2A* genes (refs 9, 16 and A. Haslinger and M.K., in preparation).

Finally, the presence of a heritable fragile site at 16q22, observed in the chromosomes of normal cells from some of these patients¹⁷⁻¹⁹, may indicate a predisposition of such patients to undergo rearrangements of chromosome 16. If the fragile site at 16q22 proves to be associated with the *inv(16)* rearrangement, *MT* will be the first gene mapped to a heritable fragile site.

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- Kagi, J. H. R. & Nordberg, M. (eds) *Experientia Suppl.* 34 (1979).
- Karin, M. & Richards, R. I. *Nature* **299**, 797-802 (1982).
- Durnam, D. M. & Palmiter, R. D. *J. biol. Chem.* **256**, 2268-2272 (1981).
- Schmidt, C. J., Hamer, D. H. & McBride, O. W. *Science* **224**, 1104-1106 (1984).
- Karin, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5494-5498 (1984).
- Harper, M. E. & Saunders, G. F. *Chromosoma* **83**, 431-439 (1981).
- Le Beau, M. M., Westbrook, C. A., Diaz, M. O. & Rowley, J. D. *Nature* **312**, 70-71 (1984).
- Le Beau, M. M. et al. *New Engl. J. Med.* **309**, 630-636 (1983).
- Karin, M. et al. *Nature* **308**, 513-519 (1984).
- Karin, M. & Richards, R. I. *Nucleic Acids Res.* **10**, 3165-3173 (1982).

- Arthur, D. C. & Bloomfield, C. D. *Blood* **61**, 994-998 (1983).
- Testa, J. R., Hogge, D. E., Misawa, S. & Zand-Parsa, N. *New Engl. J. Med.* **310**, 468-469 (1984).
- de la Chapelle, A. & Lahtinen, R. *New Engl. J. Med.* **308**, 1394 (1983).
- Gustafson, P. E. et al. *Scand. J. Haemat.* **4**, 371-379 (1967).
- Barnes, D. & Sato, G. *Cell* **22**, 649-655 (1980).
- Richards, R. I., Heguy, A. & Karin, M. *Cell* **37**, 263-272 (1984).
- Le Beau, M. M. & Rowley, J. D. *Nature* **308**, 607-608 (1984).
- Yunis, J. *Science* **221**, 227-236 (1983).
- Arthur, D. C. & Bloomfield, C. D. *Blood* **62**, Suppl. 1, 165a (1983).

Duplications of a mutated simian virus 40 enhancer restore its activity

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Enhancers are *cis*-acting control elements which can stimulate at a distance the activity of a variety of eukaryotic promoters. First identified as a repeated 72 base pair (bp) sequence upstream of the simian virus 40 (SV40) early gene promoter¹⁻⁵, enhancers have since been shown to be associated with numerous other viral⁶ and cellular⁷⁻¹³ genes. Although there are no strong homologies between the sequences of different enhancers, a number of short and degenerate consensus sequences have been identified, including the 'core' element GTGG^A/T^A/T^A/T^G (refs 14, 15) and stretches of alternating purines and pyrimidines which may have the potential to form left-handed Z DNA¹⁶. To study the functional significance of two alternating purine and pyrimidine sequences in the SV40 enhancer, we have introduced various combinations of point mutations into a modified SV40 enhancer which contained only one copy of the 72 bp element (W.H., Y.G., A. Nordheim and A. Rich, unpublished results); one of these combinations impaired both the activity of the enhancer and growth of SV40. We describe here the structure of 18 revertants of this mutant and suggest that in each of the 18 revertants, the defects of the original mutant have been overcome by simple tandem duplications in the enhancer region, all of which include the 'core' element.

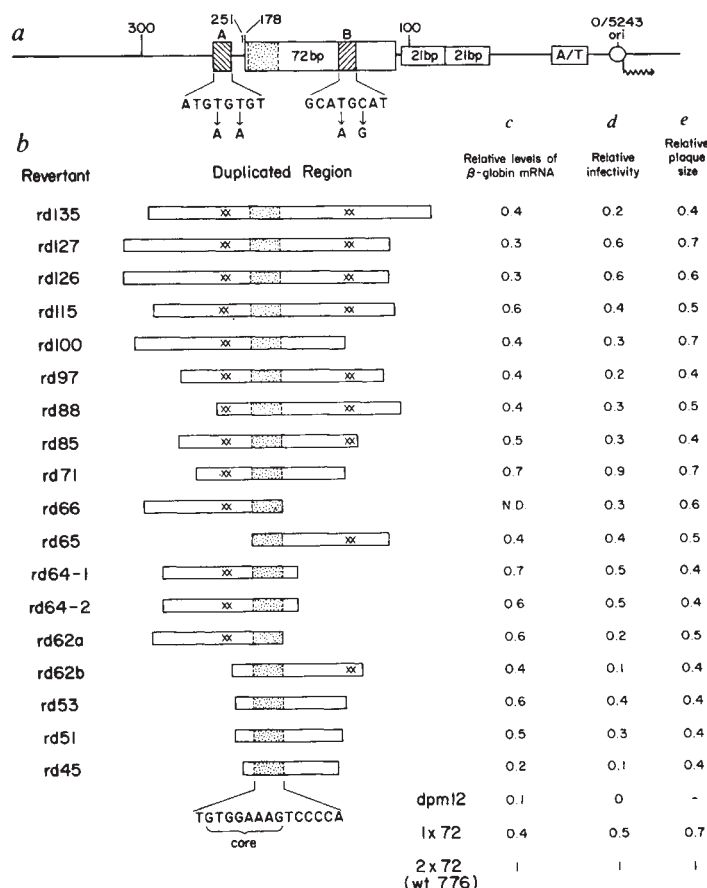
The structure of the mutant *dpm12*, from which the revertants were selected, is illustrated in Fig. 1a. The mutant differs from wild-type SV40 strain 776 in that it lacks one copy of the 72 bp repeat and contains four base substitutions: two transversions in the 8 bp stretch of alternating purines and pyrimidines upstream of the 72 bp element (hatched box A) and two transversions in the alternating purine and pyrimidine stretch in the 72 bp sequence itself (hatched box B). We have been unable to propagate this mutant in the simian cell line CV-1. Growth revertants, however, are obtained readily after transfection of CV-1 cells with relatively high concentrations of *dpm12* DNA followed by serial passage of the resulting virus stocks in CV-1 cells (see Fig. 1 legend). The enhancer sequences from revertants isolated by this method were analysed by nucleotide sequencing following molecular cloning of the enhancer region into M13 bacteriophage.

Figure 1b shows the structure of the enhancer region from 18 independent revertants; each revertant enhancer preserves the four original point mutations of *dpm12* but carries a simple tandem duplication of between 45 and 135 bp. (Tandemly duplicated regions are shown as rectangular boxes in Fig. 1b. Other than these duplications there are no changes, such as point mutations, from the *dpm12* mutant in the enhancer region of these revertants.) The revertant duplications (*rd*) are named according to their length in bp (for example, *rd135* carried a 135 bp duplication) and are shown diagrammatically in Fig. 1b in order of size. Of all the independently isolated mutants, only two (*rd64-1* and *rd64-2*) have an identical structure.

To demonstrate that these enhancer region duplications are indeed responsible for the phenotypes of the revertants, the

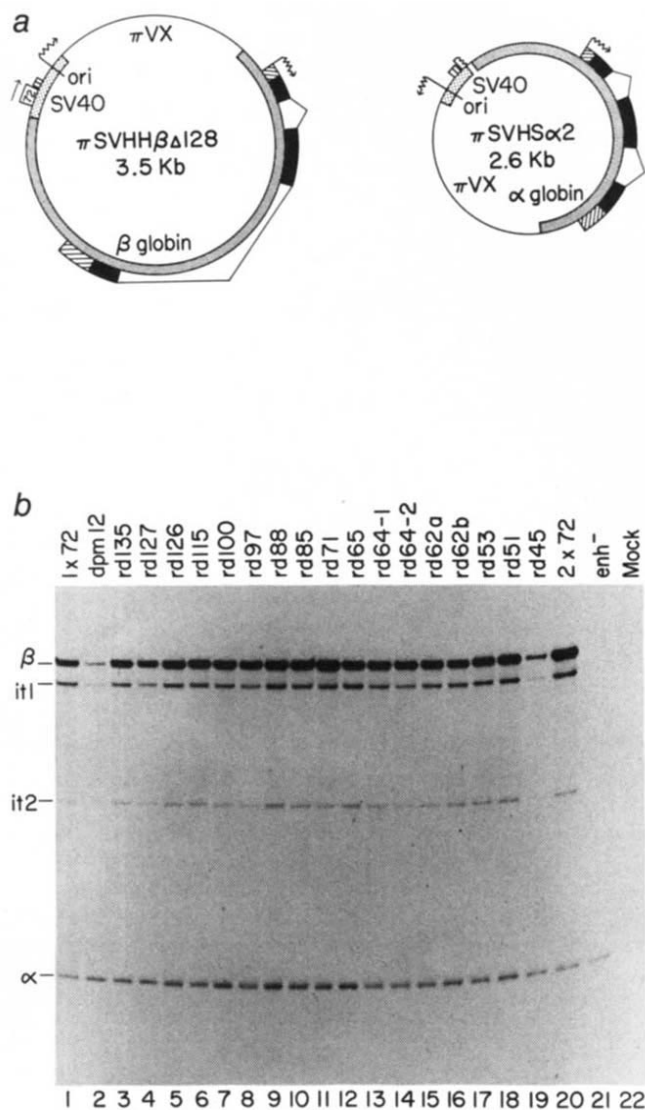
Fig. 1 Structure of revertants of the SV40 enhancer mutant, *dpm12*. **a**, Diagram of a SV40 control region containing only one copy of the 72 bp element. Shown from right to left are the early transcriptional start site (wavy arrow), the origin of replication (*ori*), the AT-rich TATA-like element (A/T), the two GC-rich 21 bp repeats and the single 72 bp element. The location of the two 8 bp stretches of alternating purines and pyrimidines is identified by hatched boxes; the sequence of each of these stretches and the base substitutions found in each of these segments in the *dpm12* mutant are shown directly below the boxes. **b**, Rectangular boxes represent the extents of the tandemly-duplicated region in each of the 18 independent revertants of *dpm12*, ordered from top to bottom by size and aligned with the diagram in Fig. 1a. The end points (from left to right) of each duplication in the SV numbering system³² are as follows (note that nucleotides 179–250 are missing from *dpm12*): *rd135*, 297–91; *rd127*, 309–111; *rd126*, 309–112; *rd115*, 295–109; *rd100*, 304–133; *rd97*, 282–114; *rd88*, 265–106; *rd85*, 283–127; *rd71*, 275–133; *rd66*, 300–163; *rd65*, 176–112; *rd64-1* and *-2*, 291–156; *rd62a*, 296–163; *rd62b*, 258–125; *rd53*, 257–133; *rd51*, 257–135; *rd45*, 253–137. The series of double Xs identifies the positions of the original point mutations where duplicated. The stippled area in each rectangular box corresponds to the 15 bp region common to all the duplications; the sequence of this region is shown at the bottom of the figure with a bracket outlining the 'core' element¹⁵. **c**, Relative concentrations of β -globin RNA after transient expression in HeLa cells of the human β -globin gene linked individually to each duplicated revertant enhancer (see Fig. 2 legend). The results are the average of three experiments and are normalized to the concentration of β -globin RNA obtained with the wild-type enhancer from strain 776, which contains two copies of the 72 bp element (2×72). **d, e**, Relative infectivity and plaque size, respectively, of SV40 containing the revertant enhancers compared with the strain 776 construct. The strain 776 pK1K1 plasmid transfection yielded 10^4 plaques μg^{-1} of SV40 DNA, 4–6 mm in diameter after 19 days. ND, not determined.

Methods. Revertant isolation and sequence analysis: The mutations in *dpm12* were made by site-directed oligonucleotide mutagenesis (ref. 33 and W.H. and Y.G., unpublished results). The plasmid pK1dpm12 contains the SV40 genome with the *dpm12* base substitutions cloned at the *EcoRI* site in the plasmid vector pMK16#6 (ref. 34). Circular *dpm12* SV40 DNA used to transfect CV-1 cells was obtained by digesting pK1dpm12 with *EcoRI* followed by treatment with ligase. Two of the revertants, *rd127* and *rd62a*, arose during the initial analyses of the various combinations of point mutations we had constructed. The remaining revertants were isolated systematically as follows: ten 25-mm Falcon plates of CV-1 cells were transfected by DEAE dextran³⁵ with 0.1 μg circular *dpm12* SV40 DNA. Virus lysates were obtained after 10 days and then passaged twice in CV-1 cells. After these passages two revertants were isolated from each virus stock by two rounds of plaque purification. DNAs from each revertant were purified by the Hirt procedure³⁶ and a portion was analysed directly by double digestion with *HpaII* and *BglII* followed by polyacrylamide gel electrophoresis. We found that in each revertant the short *HpaII/BglII* fragment spanning the enhancer region was larger than in the original *dpm12* mutant. The short *KpnI/BglII* fragment from each revertant was cloned into the M13 mp8 derivative vector mpSVO1 (W.H. and Y.G., unpublished results), carrying the SV40 *HpaII* to *HindIII* *ori* fragment and cut with *KpnI* and *BglII*. Each revertant was sequenced by the dideoxynucleotide method³⁷ as described previously³⁸, except that the reactions were carried out in microtitre plates. Because *rd127*, *rd126*, *rd100* and *rd66* had duplicated the SV40 *KpnI* site, these revertants were sequenced after cloning the *HpaII* to *HindIII* SV40 *ori* fragment from each one between the *SmaI* and *HindIII* sites of the M13 vector mp8 (ref. 39). The following pairs of revertants originated from the same transfection: *rd97* and *rd115*; *rd45* and *rd100*; *rd53* and *rd126*; *rd51* and *rd64-2*; *rd66* and *rd71*; *rd62a* and *rd88*. Revertant SV40 reconstructions and plaque assays: The enhancer region of wild-type SV40 was replaced by the revertant duplications as follows: single-stranded M13 clones containing the revertant enhancers were made double-stranded by extending an annealed sequencing primer through the cloned sequences with polymerase and deoxynucleotide triphosphates. The resulting DNAs were phenol extracted, ethanol precipitated and digested with *KpnI* and *BglII*. The short *KpnI/BglII* SV40 *ori* fragment was ligated to the large *KpnI/BglII* fragment of the plasmid pK1K1. This plasmid consists of the vector pMK16#6 and 1.27 copies of the SV40 genome in which nucleotides 346–1782 (*HpaII/EcoRI*) are duplicated (R. Gerard, personal communication). The enhancer region from the four revertants in which the unique SV40 *KpnI* site is duplicated (*rd127*, *rd126*, *rd100*, and *rd66*) was recreated in SV40 by cloning the small *KpnI* fragment from these revertants into *KpnI*-digested pK1K1dpm12 plasmid DNA and screening for clones with a single correctly-oriented small *KpnI* fragment. The duplicated 0.27 copies of SV40 sequence allow for excision and replication of the complete SV40 genome after transfection into CV-1 cells. Plaque assays were done on 60-mm plates by transfecting 40 ng DNA from each pK1K1 revertant plasmid construction into CV-1 cells; plaques were counted and their size measured on days 14 and 19. The relative results for both days were similar.



enhancer region from wild-type SV40 was replaced by the revertant duplications. The viability of the resulting constructs was then tested by plaque assay in CV-1 cells and compared with the viability of wild-type SV40. The results of these experiments are shown in Fig. 1d,e. Each duplicated revertant generates plaques after transfection of CV-1 cells under conditions where the original *dpm12* mutant is non-viable; none, however, grow as well as the wild-type SV40 strain (see 2×72 in Fig. 1). The revertants display a wide variety of infectivities ranging from *rd45* (a poorly-growing revertant) to *rd71* (with better infectivity than SV40 carrying a single copy of the wild-type 72 bp sequence; 1×72 in Fig. 1).

To establish whether the revertant phenotype is correlated with restoration of enhancer function, we measured the relative ability of each duplicated enhancer to stimulate the expression of the human β -globin gene. The plasmid constructions used for the enhancer assay are shown in Fig. 2a. The duplicated enhancers were cloned 1.2 kilobases (kb) upstream of the human β -globin gene (see Fig. 2a, left) and each construct was transfected into HeLa cells together with a plasmid carrying the human α -globin gene (π SVH α 2; Fig. 2a, right) which served as an internal control. Relative concentrations of α - and β -globin RNAs were measured by RNase protection of an internally labelled, single-stranded RNA probe after hybridization to total



cytoplasmic RNA isolated from the transfected HeLa cells.

Figure 2b shows the results of one such experiment. The revertant duplications (lanes 3-19) always yielded higher concentrations of β -globin RNA than the original *dpm12* mutant (lane 2). The relative concentrations of globin RNA were quantitated from three separate experiments (see Fig. 2 legend) and normalized to the results for the wild-type enhancer. The averaged results are shown in Fig. 1c. These revertants display a range of enhancer activities, the most active duplications (for example, *rd71* and *rd64*) exhibiting a 6- to 7-fold higher concentration of β -globin RNA than the original mutant. Although this level of enhancer activity is 30-40% less than that of the wild-type enhancer (2x72), it is better than the wild-type

Fig. 2 Transient β -globin expression assay of revertant enhancer activity. **a**, The structures of the experimental β -globin plasmid and control α -globin plasmid. The α -globin plasmid π SVHSA2 and parental β -globin plasmid π SVHS $\beta\Delta$ 128 were provided by R. Triesman (see ref. 40). Thin lines indicate π VX vector sequences; heavily stippled bands, globin sequences; bent lines (indicating intron sequences) separate the untranslated (hatched) and translated (solid) exon sequences. Lightly stippled bands designate the SV40 sequences; clear boxes, the 72 bp enhancer element and 21 bp repeats; zig-zag arrows, direction and start of transcription for both the globin and the SV40 early genes. **b**, Autoradiogram showing the levels of internally labelled α - and β -globin probe RNAs protected from RNase by cytoplasmic RNA isolated from transfected HeLa cells. In each case, the transfection involved the control α globin plasmid combined with a β -globin plasmid carrying the enhancer element indicated at the top of each lane. 1x72 and 2x72 represent the wild-type strain 776 enhancer with either one or two copies of the 72 bp element, respectively. Lane 21 (enh⁻) represents transfection with the plasmid π SVHS $\beta\Delta$ 128 (ref. 40), which lacks the SV40 enhancer region, and no DNA was used in the mock transfection (lane 22). The bands representing the correct α - and β -globin transcripts are labelled α and β respectively. Bands labelled *it1* and *it2* represent incorrect β -globin transcripts probably resulting from improper splicing⁴¹.

Methods. The π SVHH $\beta\Delta$ 128 series of plasmids containing the various SV40 enhancer elements were constructed by cloning each respective *HpaII/BglI* ori region into the plasmid π SVHS $\beta\Delta$ 128 as follows: single-stranded M13 clones were made double-stranded as described in Fig. 1 legend and digested with *EcoRI* at the M13 mp8 polylinker *EcoRI* site lying immediately adjacent to the SV40 *HpaII* site in these M13 clones. The *EcoRI* digested DNAs were made blunt-ended with polymerase and deoxynucleotide triphosphates, digested further with *BglI* and ligated to the large *PstI/BglI* fragment of π SVHS $\beta\Delta$ 128, in which, after *PstI* digestion, these ends had also been made blunt-ended. Plasmid DNAs were prepared as described previously⁴², including CsCl gradient centrifugation. The identity of the final plasmid preparations was verified by the size of the *NcoI* fragment which spans the duplications. HeLa cells at 30-50% confluency were transfected with 10 μ g of π SVHSA2 plasmid DNA and 10 μ g of the experimental β -globin plasmid DNAs by calcium phosphate coprecipitation as described previously⁴⁰. About 20 μ g cytoplasmic RNA isolated as described⁴³ 48 h after transfection were hybridized to an excess of a mixture of α - and β -globin RNA probes, prepared using SP6 RNA polymerase, followed by digestion with RNases T₁ and A as described previously⁴⁴. The plasmids pSP6 α 132 and pSP6 β 350 from which the RNA probes were generated were a gift of R. Treisman. *Bona fide* α - and β -globin mRNAs protect 132 and 350 nucleotides respectively of the appropriate probe (R. Treisman, personal communication). The RNase protected RNAs were analysed by electrophoresis through 8 M urea 6% polyacrylamide thin sequencing gels. For the experiment shown here the amount of cytoplasmic RNA used for each hybridization was adjusted so that the α -globin internal control was approximately equal in each lane. The relative RNA concentrations were quantitated from three separate transfection experiments by either densitometer scanning of films exposed without a screen or by measuring directly the radioactivity in each band; both methods gave similar results. The relative concentrations of β -globin RNA were determined by first normalizing each sample to the α -globin internal control band followed by comparison with the level of β -globin RNA in the transfection with the wild-type strain 776 enhancer.

enhancer which contains only one copy of the 72 bp element (1x72).

The structures of the *dpm12* revertants indicate that a variety of enhancer region duplications can overcome the defects created by the four point mutations. Duplications have been found also in revertants of SV40 mutants in which enhancer sequences, including either most or all of the 72 bp element, were deleted^{17,18}. In these cases, however, the recovery of enhancer activity resulted from duplication of sequences located outside the canonical enhancer region. Together, these results suggest a functional significance to the repeated motif characteristic of enhancers; in addition to SV40, repeated sequences are associated also with enhancers in BK virus¹⁹, different poly-

oma virus strains²⁰, host range mutants of polyoma virus²¹⁻²³ and retroviruses²⁴.

Unlike the SV40 mutants with a deleted enhancer region, the *dpm12* mutant retains all but four bases of the enhancer region intact. Therefore, the revertants described here allow identification of enhancer sequence elements that can best overcome the defects of an enhancer mutant. The new sequences created at the junctions of the different tandem duplications do not show any obvious homology, indicating that it is not these sequences *per se* which restore activity; in particular the patterns of alternating purines and pyrimidines destroyed by the *dpm12* mutations are not recreated by the junction sequence.

The most striking feature of the *dpm12* revertants is that a 15 bp region (the stippled areas in Fig. 1b) is shared by all the duplications, which suggests that this region plays a role in restoring activity to the *dpm12* mutant. The hypothesis is strengthened by the fact that the sequences involved in the various duplications span 147 bp; these 147 nucleotides could have accommodated the duplications 71 bp and shorter (that is, 10 of the 18 revertants) without necessarily generating any common region. The sequence of this 15 bp region, TGTGGAAAGTCCCCA, contains the 'core' element (underlined) that was first identified on the basis of related sequences that are found among many enhancer elements¹⁴. (A second consensus sequence, the 'CACA' box²⁵, overlapping the 'core' element, is contained only partially in the 15 bp common region.) The consistent duplication of the 'core' region in 18 independent revertants suggests a critical function for this element in reactivating the *dpm12* enhancer.

The structure of the *dpm12* revertants are the result of two separate constraints: the reversion phenotypes selected for and the ease with which certain types of mutation can occur. In all likelihood no point revertants were isolated both because the *dpm12* mutant carried four separate point mutations and because genomic rearrangements occur readily during SV40 replication²⁶⁻²⁸. To obtain revertants of the *dpm12* mutant we selected directly for improved growth and infectivity of SV40, rather than for enhancer function. Such a selection probably places multiple constraints on the duplications that can best restore viability to the revertants. Therefore, the duplications described here are not necessarily those in which enhancer function has been maximized. Nevertheless, each revertant displays restored enhancer activity, indicating that the major defect of the *dpm12* mutant is the lack of a healthy enhancer.

Duplications affect simultaneously two parameters: spacing between *cis*-acting elements and the sequences duplicated. Therefore, these parameters cannot be entirely independently analysed in these revertants. The three-fold difference in duplication size (45-135 bp) among the various *dpm12* revertants suggests that if spacing requirements exist they are flexible. The fact that wild-type strains of SV40 also contain enhancer duplications of various sizes is consistent with this observation; in addition to the prototype 72 bp repeat of strain 776, other wild-type strains carry duplications ranging from 64 to 93 bp in length,^{4,29-31}. Two features of the revertants, however, suggest that there is some duplication size preference: (1) those repeated sequences between 51 and 71 bp long seem to be more active as enhancers than the larger duplications (see Fig. 1c) and (2) the shortest duplication, *rd45*, is the least active of the 18 revertants.

The results described here indicate that the SV40 enhancer consists of multiple elements. The *dpm12* mutant shows that two separate regions are important for enhancer function and the revertants show that loss of enhancer function in *dpm12* can be restored by duplication of sequences not affected directly by the mutations (that is *rd45-rd53*, see Fig. 1). The consistent

duplication of the 'core' region in each of the 18 revertants suggests that these sequences are compensating for and apparently acting independently of the mutated *dpm12* sequences.

Our results show that a variety of duplications in a transcriptional control region can generate enhancers many times more active than the original. Perhaps strong viral enhancers evolved initially by duplication of weaker cellular enhancers.

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1. Banerji, J., Rusconi, S. & Schaffner, W. *Cell* **27**, 299-308 (1981).
2. Moreau, P. et al. *Nucleic Acids Res.* **9**, 6047-6068 (1981).
3. Gruss, P., Dhar, R. & Khoury, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 943-947 (1981).
4. Benoist, C. & Chambon, P. *Nature* **290**, 304-310 (1981).
5. Fromm, M. & Berg, P. *J. molec. appl. Genet.* **1**, 457-481 (1982).
6. Gluzman, Y. & Shenk, T. (eds) *Enhancers and Eukaryotic gene expression*. (Cold Spring Harbor Laboratory, New York, 1983).
7. Banerji, J., Olson, L. & Schaffner, W. *Cell* **33**, 729-740 (1983).
8. Gillies, S. D., Morrison, S. L., Oi, V. T. & Tonegawa, S. *Cell* **33**, 717-728 (1983).
9. Neuberger, M. S. *EMBO J.* **2**, 1373-1378 (1983).
10. Queen, C. & Baltimore, D. *Cell* **33**, 741-748 (1983).
11. Picard, D. & Schaffner, W. *Nature* **307**, 80-82 (1984).
12. Walker, M. D., Edlund, T., Boulet, A. M. & Rutter, W. J. *Nature* **306**, 557-561 (1983).
13. Gillies, S. D., Folsom, V. & Tonegawa, S. *Nature* **310**, 594-597 (1984).
14. Laimins, L. A., Khoury, G., Gorman, C., Howard, B. & Gruss, P. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6453-6457 (1982).
15. Weiher, H., König, M. & Gruss, P. *Science* **219**, 626-631 (1983).
16. Nordheim, A. & Rich, A. *Nature* **303**, 674-679 (1983).
17. Weber, F., de Villiers, J. & Schaffner, W. *Cell* **36**, 983-992.
18. Swimmer, C. & Shenk, T. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6652-6656 (1984).
19. Rosenthal, N., Kress, M., Gruss, P. & Khoury, G. *Science* **222**, 749-755 (1983).
20. Ruley, H. E. & Fried, M. J. *J. Virol.* **47**, 233-237 (1983).
21. Fujimura, F. K., Deininger, P. L., Friedmann, T. & Linney, E. *Cell* **23**, 809-814 (1981).
22. Sekikawa, K. & Levine, A. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1100-1104 (1981).
23. Katinka, M., Yaniv, M., Vasseur, M. & Blangy, D. *Cell* **20**, 393-399 (1980).
24. Weiss, R., Teich, N., Varmus, H. & Coffin, J. *RNA Tumor Viruses*, (Cold Spring Harbor Laboratory, New York, 1982).
25. Lusky, M., Berg, L., Weiher, H. & Botchan, M. *Molec. Cell Biol.* **3**, 1108-1122 (1983).
26. Lavi, S. & Winocour, E. *J. Virol.* **9**, 309-316 (1972).
27. Tai, H. T., Smith, C. A., Sharp, P. A. & Vinograd, J. *J. Virol.* **9**, 317-325 (1972).
28. Martin, M. A., Gelb, L. D., Fareed, G. C. & Milstein, J. B. *J. Virol.* **12**, 748-757 (1973).
29. Reddy, V. B. et al. *Science* **200**, 494-502 (1978).
30. Ghosh, P. K. et al. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1386-1390 (1981).
31. Been, M. D., Burgess, R. R. & Champoux, J. J. *Nucleic Acids Res.* **12**, 3097-3114 (1984).
32. Tooze, J. (ed.) *DNA Tumor Viruses*. (Cold Spring Harbor Laboratory, New York, 1981).
33. Zoller, M. J. & Smith, M. *Nucleic Acids Res.* **10**, 6487-6500 (1982).
34. Gluzman, Y. & Ahrens, B. *Virology* **123**, 78-92 (1982).
35. McCutchan, J. H. & Pagano, J. S. *J. natn. Cancer Inst.* **41**, 351-357 (1968).
36. Hirt, B. *J. molec. Biol.* **26**, 365-369 (1967).
37. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
38. Biggin, M. D., Gibson, T. J. & Hong, G. F. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3963-3965 (1983).
39. Messing, J. & Vieira, J. *Gene* **19**, 269-276 (1982).
40. Treisman, R., Green, M. R. & Maniatis, T. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7428-7432 (1983).
41. de Villiers, J. & Schaffner, W. *Nucleic Acids Res.* **9**, 6251-6264 (1981).
42. Ish-Horowitz, D. & Burke, J. F. *Nucleic Acids Res.* **9**, 2989-2998 (1981).
43. Berk, A. J. & Sharp, P. A. *Cell* **12**, 721-732 (1977).
44. Zinn, K., DiMaio, D. & Maniatis, T. *Cell* **34**, 865-879 (1983).

Erratum

Involvement of circular intermediates in the transfer of T-DNA from *Agrobacterium tumefaciens* to plant cells

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Nature **313**, 191-196 (1985)

This article was published with the sixth author's surname spelt incorrectly. It is shown correctly above.

High priests of precision

William H. Press

The Fundamental Physical Constants and the Frontier of Measurement.

By B.W. Petley.

Adam Hilger: 1984. Pp.346. £28.50, \$49.

IN A by now time-worn story, an experimental physicist is asked by a pure mathematician to name a few prime numbers. The physicist replies that 3, 5 and 7 are prime, 9 seems not to be, and 11 and 13 are prime — “hmm...nine must be experimental error”. The fact that this is (was once?) funny reveals, I think, a couple of assumptions about science that we take for granted and rarely examine. First is the belief that mathematics is “exact”, so that the very notion of experimental error is laughable. Second, more subtle, is the implicit assumption that physics can be made arbitrarily precise, revealing an underlying reality of fully mathematical exactitude: we little doubt that the experimental physicist in the story feels that he can build a better apparatus, eliminate the experimental error, and prove that 9 is a prime! To put it another way, I think that the joke loses some of its pith if “experimental psychologist” or “anthropologist” is substituted for “experimental physicist”, and I mean this as no insult to my social-scientist colleagues, whose fields do not (and have no need to) pretend to the exactitude of physics.

In our time, only a small fraction of physicists work to uphold their discipline's reputation as an “exact” science. Search not for such individuals among the high-energy particle physicists at CERN, where important discoveries tend to be qualitative, as confirming the existence of the theoretically-predicted top quark or intermediate vector boson. Nor look, generally, to the solid-state physicists at Bell Labs; there too, the most interesting discoveries are qualitatively new material behaviours or transitions. For exact physics, look instead to Britain's National Physical Laboratory (where Dr Petley works), or, in the United States, to the National Bureau of Standards or affiliated Joint Institute for Laboratory Astrophysics, or to the laboratories of individual experimenters in small or large universities. These are the habitats of the “metrologist”, whose intellectual world is the subject of Dr Petley's book.

Metrology is the physics of high-precision measurement. Is it truly fundamental science, or is it merely necessary, industrial standards-keeping? That question runs between the lines of this book. Not unexpectedly Dr Petley holds a brief on one side. Occasionally one finds his tone a bit too defensive, a bit too revealing of the fact that the metrologist, whether unfairly or

not, ranks low in today's physics pecking order. The author's case against this prejudice is strongest not when he waxes intellectual about the matter, but rather when he describes the elegance and diversity of modern, high-precision experiments.

The question “fundamental or not” has some subtlety. A simple example is the speed of light. Special relativity, that essential framework on which all of modern physics rests, predicts and requires the speed of light c to be absolutely

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constant, independent of where and how it is measured. In this light, high-precision measurement of c seems fundamental indeed. Speed being a length per time, one must “bring” the standard second and standard metre to any laboratory in which c is to be measured. The second (that is, the first) is relatively transportable, exemplified in caesium-133 atomic clocks and hydrogen masers. The metre has historically proved more difficult to transport or, for that matter, define. Its definition as a platinum-iridium bar in Paris gave way in 1960 to a certain number of wavelengths of krypton-86 light. During the 1970s the overriding limitation on the measurement of c , whose value was now known to be something like $299,792,458.1 \pm 1.9 \text{ m s}^{-1}$, became the accuracy with which a laboratory baseline length could be related to the krypton-86 standard. In 1983 the speed of light became defined as $299,792,458 \text{ m s}^{-1}$; the metre lost its independent definition and is now derived from the standard of time. But consider the plight of the experimenter who was thought to be measuring c , which seemed fundamental, but is now revealed as having merely measured the standard metre, which sounds terribly industrial!

In this book one is plunged back and forth between the worlds of physics and international commerce: “Although it is tempting to set up a system of electrical

units involving fundamental constants, for example, the Josephson effect for the volt and the quantised Hall conductance for the siemens, or ohm, such systems must be set up with care. If they are not, one finds that the [magnetic permeability of vacuum becomes] a constant which must be evaluated experimentally, *as it did during the 1930s for a while*”. (Emphasis added.) For a while?! This is not ordinary science.

It is an arcane world, and Dr Petley is a fascinating guide, in spite of (or perhaps because of) his tendency to dive enthusiastically into a welter of details which will leave non-physicist readers gasping for breath — but awestruck. If “God is in the details”, then the high-precision metrologist is surely above the saints. Physicist readers will be able to follow the exposition, even when it is occasionally muddy, and will be repaid by learning a lot.

The book is organized into a couple of

Record speed — the microwave interferometer used by K.D. Froome to measure the velocity of light at the National Physical Laboratory in 1957. His result formed the basis for the value recommended by the International Astronomical Union until it was superseded by the measurements which culminated in the redefinition of the metre in 1983.

introductory and philosophical chapters, followed by individual chapters on the “mechanical” and the “electrical” fundamental constants. There is a chapter on high-precision tests of quantum electrodynamics (most notably the Lamb shift), one on matters relating to gravitation, and one on so-called “null experiments” which test experimentally such tacit (and often over-looked) assumptions as that a body's inertia is the same no matter what the direction in which it is accelerated. Also included is discussion of some trendy topics, such as the large-number coincidences in cosmology and the prospects for detecting gravitational waves. But in truth these are superfluous, since the author is at his best when guiding us through real-world laboratory details: “Consequently, by setting on the side of this peak it was possible to check that the displacement of the crystal was in a straight line to ± 0.00006 ”. In fact, there was a small curvature of $0.0017''$ which was taken into account...”. Read on, and you will learn how the “Siegbahn constant” is measured! It gives a precise calibration of the lattice spacing in silicon, needed in turn for accurate determination of the Avogadro constant so fundamental to physical chemistry. □

William H. Press is Professor of Astronomy and of Physics at Harvard University.

Saturn celebration

D.J. Southwood

Saturn.

Edited by Tom Gehrels and Mildred Shapley Matthews.

University of Arizona Press: 1984.
Pp. 968. \$37.50.

FOR many years planetary science was at the core of astronomy. In the early and middle parts of the twentieth century it drifted to the periphery, but then returned to the centre of public perception of the subject with the advent of the space era. A peak of interest must have been achieved with the Voyager encounters with Jupiter and Saturn in 1979–1981. One worries now that the sense of wonder seems to have been lost. Whether any mission will return to the Saturnian system before the next century must be doubtful. In such a light a book entitled *Saturn* must be looked at with interest. But while it may need to remain in print for a long while, will it remain in demand?

In a scientific research environment where the future is financially uncertain, with small-mindedness engendered by funding strictures on both sides of the Atlantic, one of the weaknesses of planetary science is its multidisciplinary nature. For better or worse this book fits in with the spirit of its subject. It is broad, demanding and calls on knowledge of all branches of the planetary and geosciences.

The book is very well produced (with aid from NASA) and is reasonably priced. The authors, 78 in number, have been chosen well. Stone and Owen introduce matters

with a helpful overview whose structure mirrors the rest of the book, the topics breaking down as follows: the planet itself (interior, atmospheric composition, structure and dynamics, clouds, upper atmosphere, ionosphere); its magnetosphere (magnetic field, energetic charged particles, plasma and plasma waves, radio waves); and the rings and satellites (with Titan singled out for special attention). The introduction is then followed by a brief, instructive history (by Van Helden) of Saturn as examined through the telescope. This account should be required reading for any astronomer, if only for the light thrown on the relationship between theory and experiment in a subject where observational facts are sparse.

We can now say, however, that observational facts on the Saturnian system are no longer sparse, and the remainder of the book (900 pages) contains the technical details. Some of the material is very familiar to me, some of it far from my area of expertise. Yet although presentation varies from paper to paper, none of the contributions is inaccessible; to me this indicates the book is set to be a major source for years to come.

It seems futile to attempt to select highlights in the collection. To do so misses the point; the book's strength is its breadth of coverage. Buy it for that. Much of the material should be useful even at undergraduate level, and planetary scientists will not want to be without it — would that all astronomers and geophysicists thought they should not be without it. □

D.J. Southwood is a Reader in the Department of Physics at Imperial College, University of London.

clever computer program; it was the beginning of a technology and is at the heart of whatever credibility AI and the "Fifth Generation" currently have. The program started out as Edward Shortliffe's PhD research but at various times involved a total of 30 people on as many projects. It deserves a careful appraisal. Buchanan and Shortliffe's volume looks back over the decade of the project's existence to try to provide both a historical record and a properly digested assessment.

The book is a collection of 36 papers covering numerous facets of the development of MYCIN. History and design of the original system are described, and then a series of sections deals with subsidiary themes, many of which have come to be core areas for the development of expert systems generally. Techniques for representing knowledge of different types and for different tasks are described, along with issues about building and checking knowledge bases. The use of the knowledge base for explanation and for teaching, as well as for advice, is extensively discussed. The problems of managing uncertainty, and of generalizing MYCIN's inference and knowledge representation techniques to cover other domains are analysed. A particular strength of the book is the emphasis on transferring the technology out of the research laboratory, reflected in sections on performance evaluation and on acceptability.

Many papers in the collection have appeared elsewhere, but the range of publications is wide, and the period long, so this anthology is fully justified. Furthermore, much of the material has been specially prepared, and many of the original manuscripts revised and brought up to date. The chance to analyse an influential development carefully and self-critically is infrequently offered in technology; here it has been taken.

What is the significance of the MYCIN project? In fact MYCIN is not in routine use, though it inspired systems which are. If software could be put in a museum, MYCIN would hold the same position as *The Rocket* in the National Railway Museum. The project is also an important example of how high technology research should be done — with flair and ingenuity, but with an eye on detail and systematic investigation, and on the way that the technology will be used. Newell thinks the MYCIN project also exemplifies the science of AI, by which he means the empirical exploration of intelligent systems. I am not so sure; I think that MYCIN has told us little that we did not know about intelligence and its principles. What MYCIN has shown us is how to put something that resembles rudimentary, but useful, intelligence into a machine, and that is more than enough. □

John Fox is Head of the Biomedical Computing Unit at the Imperial Cancer Research Fund Laboratories, London.

In the beginning

John Fox

Rule-Based Expert Systems: The MYCIN Experiments of the Stanford Heuristic Programming Project.

Edited by Bruce G. Buchanan and Edward H. Shortliffe.

Addison-Wesley: 1984. Pp. 748.
\$39.95, £39.

EDWARD Feigenbaum of the Heuristic Programming Project at Stanford University, and author of a controversial book about the "Fifth Generation" in computing, believes that artificial intelligence is not just another computer revolution but *the big one*. The stimulus for such a confident claim was the announcement by Japan, in 1981, of its plans to build a new generation of computers based on the hardware and software technology of AI. This book is about one of the crucial developments in AI software, some would say *the* crucial development, the advent of expert systems.

Expert systems are programs which have enough expertise in specialist fields that they can solve problems at a level comparable to that of a human expert. Opinions vary as to which program was the first true expert system, but I am inclined to agree with Allen Newell, the eminent pioneer of AI who writes a foreword to the book, that "MYCIN is the original expert system that made it evident to all the rest of the world that a new niche had opened up".

The function of MYCIN was to advise doctors on the nature of bacterial infections and the use of antibiotics. Many of the features that it introduced are now seen as pivotal to the concept of expert systems, notably the emphasis on facts and rules of good judgement (rather than formal, quantitative methods) and the ability to explain the advice given. Another pattern for the future was the later development of EMYCIN, or Essential MYCIN, a package stripped of its specialist medical knowledge to give a general purpose "shell" which can be (and has been) used to build expert systems for other applications.

MYCIN was more than just another

Active in reducing tension

Martin J. Schick

Surfactants.

Edited by Th. F. Tadros.

Academic: 1984. Pp.342. \$30, £20.

SURFACTANTS not only present intriguing problems of basic science to the physical chemist but are of widespread application in industry. The field is thriving, on both its "pure" and "applied" sides. To examine the current state of work in this area, a residential school — sponsored by the Royal Society of Chemistry and attended by a galaxy of experts — was held at Bristol University in 1983. This book contains the printed record of lectures presented at the meeting, and is divided into two unequal parts; ten chapters deal with the basic principles of the physical chemistry of surfactants and dispersed systems, while a final three cover applications.

The first four contributions provide a contemporary overview of the solution properties of surfactants, with accounts of the thermodynamics of surfactant solutions, phase equilibria and mesophases in surfactant systems, and the structural aspects of micellar systems. The last of these, by B. Lindman, is especially successful in making clear the importance of simple geometric features and electrostatic effects. New methods for the study of micellar systems, such as quasi-electric light scattering, multifield ^{13}C NMR chemical shift, multifield proton NMR and NMR water self-diffusion, are explained, and the timely subject of microemulsions is illustrated by J.T.G. Overbeek with phase diagrams, thermodynamics, techniques of characterization and applications. Also included are discussions of the microstructure and rheology of surfactants in dilute and concentrated systems, adsorption at the three interfaces (with explanations of experimental procedures), and the role of surfactants in controlling the stability of emulsions, foams and suspensions. This first part of the book concludes with an up-to-date account of the fundamentals of wetting, incorporating a selective review of the recent literature.

Unfortunately, the last three contributions — on surfactants in enhanced oil recovery, macromolecular surfactants, and applications in biological systems — do not measure up to the standard of the earlier part of the book. For example, the title "Macromolecular Surfactants" is grossly misleading since this chapter deals almost exclusively with non-ionic surfactants originating only from the author's own company. On p.287 the misprint of the formula of the alkylphenol ethoxylate is unfortunate and on p.305 the downgrading of the HLB system is not justified.

Yet the high quality of the first ten chapters more than compensates. The concise treatment and scholarly approach to be found there will make this volume useful to both the newcomer and the practitioner who wants to learn about the latest developments. Graduate and research chemists will benefit greatly from reading it. Still, I can't help but feel that omission of the applications chapters and a title such as *Introduction to the Physical Chemistry of Surfactants* would have meant an altogether more useful book. □

Martin J. Schick is a consultant physical chemist based in New York and editor of the *Surfactant Science series* published by Marcel Dekker.

Something cooking

G.D. Clarke

Cell Culture Methods for Molecular and Cell Biology, Vols 1,2,3 and 4.

Edited by David W. Barnes, David A. Sirbasku and Gordon H. Sato.

Alan R. Liss/Wiley: 1984. Vol. 1 pp.353, \$49.50, £38. Vol. 2 pp.280, \$39.50, £30. Vol. 3 pp.300, \$49.50, £38. Vol. 4 pp.263, \$39.50, £30.

THE science of making media for animal cell culture grew slowly out of the black arts of the bacteriologist's "kitchen" with its steamy atmosphere, arcane recipes and horny-handed aficionados. They knew nothing of the composition of their brew, merely that it worked. This art, even in its modern, scientific form is not an activity with appeal for all, yet scientists in the laboratories of such as Richard Ham and Gordon Sato — herein represented — have made contributions which show signs of increasing application, not only to pure scientific enquiry, but also to industrial and clinical practice. Their achievement is in devising serum-free media which do not merely grow aberrant cells selected in these media, but also cells from embryonic, adult and tumour tissues of many different types.

These are not books for every biologist or the common reader: Alan Freshney's recent *Culture of Animal Cells* from the same stable fulfils that role best. These are for the specialist and, in particular, for those who choose not to rely on commercial sources for the many growth factors which now take the place of serum. Much of the first volume concerns the history and philosophy of synthetic media and solid substrata, though it also contains details for the purification of the less esoteric growth factors (PDGF, MSA, EGF, type β -TGF, MDGF and ECGF). Others of more limited use are conveniently covered in the more specialized volumes: FGF in Vol. 3 (*Epithelial Cells and Fibroblasts*), interleukin-2, T-cell growth factor and thymosins in Vol. 4

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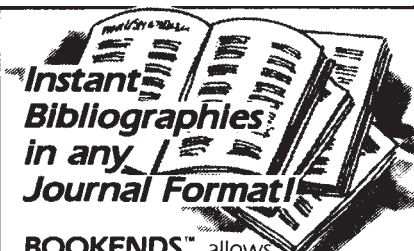
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(*Neuronal and Lymphoid Cells*). Volume 2 deals with *Cells of the Endocrine System*.

Why would one wish to grow cells in defined media? Well, for instance, to grow cells other than the enduring fibroblast, when methods involving selective toxicity have failed; and, having such cells, to find what factors will support their survival and growth, though any extrapolation from "can" *in vitro* to "do" *in vivo* is dangerous. It is ironic that "organ" culture techniques have used synthetic media for three decades to study differentiated function. Now that purified factors are available for the study of cellular interactions, the technique no longer commands much interest.

Defined media can also be used to identify and purify molecules secreted by particular cells. When cells are grown from a patient's own skin for transplantation, such media may have advantages — will the time come, perhaps, when cells grown in culture can be used to relieve genetic deficiencies? In any cultures of this kind the choice of solid substrata arises (Vol. 1). But currently the main use for defined media is for the growth of hybridomas,

allowing easier purification of monoclonal antibodies than when cells are grown in animals. If human hybridomas are used this method would be essential. Monoclonal antibodies are of potential application throughout the whole of biology and Vol. 4 is, thus, likely to be of greatest use.

Because serum proteins can mask many toxic agents and, to a degree, protect against the mutual inhibition of some reagents and contamination from the operator, the more defined media present greater difficulties. Iscove's troubleshooting list (Vol. 4) could well have been repeated in the other volumes.

These volumes cover a wide range of techniques of limited application. As all their advice is already in specialist journals, only establishments where cell culture is in wide use would find them invaluable and might need all four. Single chapters might provide unexpected help to individual cell or molecular biologists. □

G.D. Clarke recently retired from the scientific staff of the Imperial Cancer Research Fund Laboratories, London.

Streams of cases and materials

Paul Sieghart

Law, Science and Medicine.

By Judith Areen, Patricia A. King, Steven Goldberg and Alexander Morgan Capron.

Foundation Press, 170 Old Country Rd, Mineola, New York 11501: 1984. Pp.1,494. \$34.50.

LAW, science and medicine have interacted for as long as they have existed as separate disciplines. Rather more recently, their practitioners have grown increasingly ignorant of what others are up to, both within each discipline and across them. Much more recently still, this mutual ignorance has come to be seen as a threat to our civilized values — if not our collective survival — rather than just an irritant, and that perception is now spawning a growing literature.

One therefore turns expectantly to a 1,500-page volume with this title, hoping to find a full and structured analysis and discussion. Expectations rise even further when one sees that the volume is organized into four parts — I, The Evolving Relationship of Science and Medicine to the Law; II, Controlling Science and Medicine: the Roles of Individuals, Groups and the State; III, Achieving the Goals of Public Policy: Avoiding Harm and Promoting Fairness; and IV, Exploring Problems at the Frontier. Yes, a seemingly ordered and interesting approach, outlining a survey supported

by arguments in progressive steps, within a directed sweep from the past to the future.

Then there is an expanded table of contents of no less than 22 pages, followed by a table of cases and another of authorities. Splendid. And then, with an icy shock, all expectations are dashed. There is no analysis, and no discussion. There is no order and no interest. There is no survey, no argument, no progression, no steps and no sweep. Instead, one is faced with yet another of those vast but useless compilations which go under the generic title of *Cases and Materials on . . .*: a veritable magpie's nest of clips from other people's writings, for the assembly of which all that is needed is a sharp pair of scissors and a plentiful supply of paste.

Time was, indeed, when case-books had their uses. In the Middle Ages, English law reporters published Year Books, to keep their colleagues up to date with what the judges were deciding. Later, they kept commonplace books, in which they noted down passages they thought worth remembering. But that was long ago; today, in the law, we have official law reports which record the primary data, and textbooks which order, analyse and discuss them. If you want to know the current state of the art on home-made wills, you consult the latest edition of *Williams on Wills*; if this does not give you the answer, you read the original reports of the decisions which the current editor of *Williams* has collected in copious references to his synoptic text. And what goes for home-made wills goes likewise for solid-state physics, organic chemistry, bacteriology or any other area of science.

But the modern case-book does none of

these things. Neither its index nor its content will help you to discover the state of the art about anything. Instead, it is a short cut for the hard-pressed legal academic to publish without either perishing or having to think, and it is bought by a captive market of other hard-pressed legal academics who are given no time to think before they have to teach. Books such as these contain no more than random chunks of other people's work — from philosophers to judges to novelists — followed on occasions by a set of questions presumably intended for classroom discussion, of which this is a random but typical example from the present work:

Hoffman's description of Einstein's development of the special theory of relativity provides a glimpse of a great scientific mind at work. Would Einstein's undoubted brilliance have made him a successful judge? Indeed, is brilliance, as contrasted with wisdom or empathy, a necessary characteristic of a good attorney or judge?

We find this on p. 197, in the section on "The Scientific Basis of Nuclear Energy", though we are not told what that is, or what it has to do with the passage just cited. But try throwing it at your students, and with any luck their credits may redound to your credit.

My bias against case-books will by now have become clear. But let us assume that there could be such a thing as a good and useful case-book, and then see whether the present member of the genus falls within that category. Try, for instance, a simple standard test: look for your own name in the table of authorities. Heavens, it's *there*! Not only that, but its typeface ranks it in importance above Dworkin, Nozick, Rawls, Popper and Pope Pius XII. It still reads well, too. But under what heading do we find it? "The Social Responsibility of the Scientist", which was its subject? Not at all: "The Development and Impact of Genetic Engineering". How strange — considering that the paper had nothing to say on that mighty subject, and was published two and a half years before the Asilomar conference.

One might not have guessed that, mind you, from the reference given: 1 *Hastings Cntr. Studies* 7-16. In fact, that was a later (and incomplete) reprint. The paper was first published in *Nature* in 1972, but that reference is omitted — perhaps because the book confines itself strictly to American "cases and materials", for all the world as if no one elsewhere had ever had anything useful to say on these matters.

What was it the Romans used to say long ago? *Melius est petere fontem quam sectari rivulos* — it is better to go to the fountain-head than to follow the streams. That goes for all scholarship, even case-books. □

Paul Sieghart is a retired barrister. His 1972 paper in Nature led to the foundation of the Council for Science and Society, of which he was the first Vice-Chairman.

Physics

- Fusion Energy.** By ROBERT A. GROSS. Wiley: 1984. Pp. 328. ISBN 0-47188470-7. Np.
- Lectures on the Electrical Properties of Materials, 3rd Edn.** By L. SOLYMAR and D. WALSH. Oxford University Press: 1984. Pp. 413. ISBN 0-19-851163-9. £20.
- Magneto-Solid Mechanics.** By FRANCIS C. MOON. Wiley: 1984. Pp. 436. ISBN 0-471-88536-3. £31.65.
- NMR of Newly Accessible Nuclei, Vol. 1.** PIERRE LASZLO (ed.). Academic: 1983. Pp. 298. ISBN 0-12-437101-9. \$59.00.
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- The Thorny Way of Truth Part II: Documents on the Invention of the Perpetuum Mobile, on the Centurial Blindness of Mankind, and on their Frantic Perseverance in It.** By STEFAN MARINOV. East-West International Publishers, Niederschöcklstr. 62, 8044 Graz, Austria: 1984. Pp. 285. \$25.

Chemistry

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- Chemistry Reviews, Vol. 5.** M.E. VOL'PIN (ed.). Harwood Academic: 1984. Pp. 397. ISBN 3-7186-0137-0. \$170.
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Environmental Sciences

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Earth Sciences

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- Quaternary Coastlines and Marine Archaeology: Towards the Prehistory of Land Bridges and Continental Shelves.** P.M. MASTERS and N.C. FLEMMING (eds). Academic: 1983. Pp. 641. ISBN 0-12-479250-2. £43.

- Terra Non Firma: Understanding and Preparing for Earthquakes.** By JAMES M. GERE and HARESH C. SHAH. W.H. Freeman: 1984. Pp. 203. Hbk ISBN 0-7167-1496-5; pbk ISBN 0-7167-1497-3. Hbk £19.95; pbk £11.95.

Biological Sciences

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- Biology of the Integument. 1: Invertebrates.** J. BEREITER-HAHN, A.G. MATOLTSY and K. SYLVIA RICHARDS (eds). Springer-Verlag: 1984. Pp. 841. ISBN 3-540-13062-4/0-387-13062-4. DM 298, \$116.80.
- Biotechnology in the Marine Sciences.** RITA R. COLWELL, E. RAY PARISER and ANTHONY J. SINSKEY (eds). Wiley: 1984. Pp. 292. ISBN 0-471-88276-3. £38.35.
- The Birds of the Wetlands.** By JAMES HANCOCK. Croom Helm: 1984. Pp. 152. ISBN 0-7099-1287-0. £13.95.
- Caffeine.** PETER B. DEWS (ed.). Springer-Verlag: 1984. Pp. 260. ISBN 3-540-13532-4. DM 88, \$32.10.
- Cartilage. Vol. 1 Structure, Function, and Biochemistry, Vol. 3 Biomedical Aspects.** BRIAN K. HALL (ed.). Academic: 1984. Vol. 1 pp. 385, ISBN 0-12-319501-2; Vol. 3 pp. 353, ISBN 0-12-319503-9. Vol. 1 \$55, Vol. 3 \$49.50.
- Catecholamines 3 Vols.** Neurology and Neurobiology, Vols 8A, 8B and 8C. EARL USDIN et al. (eds). Alan R. Liss: 1984. Vol. 8A pp. 420, ISBN 0-8451-2707-1, £91. Vol. 8B pp. 520, ISBN 0-8451-2708-X. Vol. 8C pp. 260, ISBN 0-8451-2709-8. £72.
- Cell and Tissue Regeneration: A Biochemical Approach.** By MARGERY G. ORD and LLOYD A. STOCKEN. Wiley: 1984. Pp. 221. ISBN 0-471-86248-7. £42.95.
- Chloroplasts.** By J. KENNETH HOOBER. Plenum: 1984. Pp. 280. Hbk ISBN 0-306-41643-3; pbk ISBN 0-306-41686-7. Hbk \$42.50; pbk \$19.95.
- Class Experiments in Plant Physiology.** By HANS MEIDNER. George Allen & Unwin: 1984. Pp. 169. Hbk ISBN 0-04-581015-X; pbk ISBN 0-04-581016-8. Hbk £20; pbk £9.96.
- Cloning and the New Genetics.** By MARGARET O. HYDE and LAWRENCE E. HYDE. Enslow, Bloy Street, Box 777, Hillside, NJ 07207: 1984. Pp. 128. ISBN 0-89490-084-6. \$10.95.
- Comparative Pathobiology. Vol. 6 Invertebrate Blood.** THOMAS C. CHENG (ed.). Plenum: 1984. Pp. 211. ISBN 0-306-41674-3. \$45.
- Comparative Pathobiology. Vol. 7 Pathogens of Invertebrates.** THOMAS C. CHENG (ed.). Plenum: 1984. Pp. 278. ISBN 0-306-41700-6. Np.
- Contemporary Problems in Plant Anatomy.** RICHARD A. WHITE and WILLIAM C. DICKSON (eds). Academic: 1984. Pp. 598. ISBN 0-12-746620-7. \$49, £38.

Current Topics in Microbiology and Immunology. Retroviruses 3. P.K. VOGT and H. KOPROWSKI (eds). Springer-Verlag: 1984. Pp.115. ISBN 3-540-13307-0/0-387-13307-0. Np.

Cyclic Nucleotides in the Nervous System. By GEORGE I. DRUMMOND. Raven: 1984. Pp.135. Pbk ISBN 0-88167-015-4. Pbk \$20.50.

The Cytoskeleton: Cellular Architecture and Choreography. By ALICE B. FULTON. Chapman & Hall: 1984. Pp.80. Pbk ISBN 0-412-25510-3. Pbk £3.25.

The Development of the Rat Spinal Cord. By JOSEPH ALTMAN and SHIRLEY A. BAYER. Springer-Verlag: 1984. Pp.166. Pbk ISBN 3-540-13119-1. Pbk \$23.60, DM60.

Developments in Biological Standardization, Vol. 56: Brucellosis. L. VALETTE and W. HENNESSEN (eds). Karger: 1984. Pp.779. ISBN 3-8055-3944-4. SwFr. 160, DM 192, US\$ 96.00.

Diseases of Amphibians and Reptiles. GERALD L. HOFF, FREDERIC L. FRYE and ELLIOTT R. JACOBSON (eds). Plenum: 1984. Pp.784. ISBN 0-306-7111-1. \$97.50.

DNA Repair and Its Inhibition. A. COLLINS, C.S. DOWNES and R.T. JOHNSON (eds). IRL: 1984. Pp.371. Pbk ISBN 0-904147-73-8. Pbk £27, \$55.

Dopamine Receptor Agonists. GEORGE POSTE and STANLEY T. CROOKE (eds). Plenum: 1984. Pp.388. ISBN 0-306-41654-9. \$55.

Ecology and Evolutionary Biology: A Round Table on Research. GEORGE W. SALT (ed.). University of Chicago Press: 1984. Pp.130. Pbk ISBN 0-226-73443-9. Pbk £6.75.

The Ecology of Tropical Food Crops. By M.J.T. NORMAN, C.J. PEARSON and P.G.E. SEARLE. Cambridge University Press: 1984. Pp.369. Hbk ISBN 0-521-24082-4; pbk ISBN 0-521-28428-7. Hbk £32.50; pbk £12.95.

Fish Migration. By BRIAN A. McKEOWN. Croom Helm: 1984. Pp.224. ISBN 0-917304-99-3. £19.95.

Fish Physiology, Vol. X. Gills. W.S. HOAR and D.J. RANDALL (eds). Academic: 1984. Pp.456. ISBN 0-12-350430-9. \$59, £41.50.

Folate Antagonists as Therapeutic Agents: Biochemistry, Molecular Actions, and Synthetic Design. F.M. SIROTNAK *et al.* (eds). Academic: 1984. Pp.367. ISBN 0-12-646901-6. \$69.50, £49.

Gene Expression: The Translational Step and Its Control. BRIAN F.C. CLARK and HANS UFFE PETERSEN (eds). Munksgaard: 1984. Pp.527. ISBN 87-16-09561-8. D.Kr. 350.

Applied Biological Sciences

Distribution, Biology, and Management of Exotic Fishes. By WALTER R. COURTENAY Jr and JAY R. STAUFFER Jr. Johns Hopkins University Press: 1984. Pp.430. ISBN 0-8018-3037-0. \$40.

Dual Addiction: Pharmacological Issues in the Treatment of Concomitant Alcoholism and Drug Abuse. MARY JEANNE KREEK and BARRY STIMMEL (eds). Haworth: 1984. Pp.120. ISBN 0-86656-318-0. \$22.95.

Endoscopic Interpretation: Normal and Pathologic Appearances of the Gastrointestinal Tract. By MICHAEL O. BLACKSTONE. Raven: 1984. Pp.591. ISBN 0-89004-789-8. \$130.

Etosopide (VP-16) Current Status and New Developments. BRIAN F. ISSELL *et al.* (eds). Academic: 1984. Pp.355. ISBN 0-12-375350-3. \$30, £21.

Folate Antagonists as Therapeutic Agents. F.M. SIROTNAK *et al.* (eds). Academic: 1984. Pp.303. ISBN 0-12-646902-4. \$59.50, £42.

Frontiers in Biochemical and Pharmacological Research in Depression. Advances in Biochemical Psychopharmacology, Vol. 39. EARL USDIN *et al.* (eds). Raven: 1984. Pp.482. ISBN 0-89004-243-8. \$93.50.

Gene Transfer and Cancer (Progress in Cancer Research and Therapy, Vol. 30). M.L. PEARSON and N.L. STERNBERG (eds). Raven: 1984. Pp.398. ISBN 0-89004-899-1. \$76.00.

Handbook of Plant Cell Culture, Vol. 2 Crop Species. W.R. SHARP *et al.* (eds). Collier Macmillan: 1984. Pp.644. ISBN 0-02-949780-9. £49.50.

Hepatitis B: The Virus, the Disease, and the Vaccine. IRVING MILLMAN, TOBY K. EISENSTEIN and BARUCH S. BLUMBERG (eds). Plenum: 1984. Pp.251. ISBN 0-306-41723-5. \$39.50.

Hormones and Cancer 2. Progress in Cancer Research and Therapy Vol. 31. FRANCESCO BRESCIANI *et al.* (eds). Raven: 1984. Pp.735. ISBN 0-88167-031-6. \$88.50.

Human Trophoblast Neoplasms. Advances in Experimental Medicine and Biology, Vol. 176. ROLAND A. PATILLO and ROBERT O. HUSSA (eds). Plenum: 1984. Pp.505. ISBN 0-360-41735-9. Np.

Hypertension: Physiological Basis and Treatment. HELEN H. ONG and JOHN C. LEWIS. Academic: 1984. Pp.327. ISBN 0-12-526850-5. \$35.

The Importance of Laboratory Animal Genetics, Health, and the Environment in Biomedical Research. EDWARD C. MELBY Jr and MELVIN W. BALK (eds). Academic: 1984. Pp.284. ISBN 0-12-489520-4. \$25.

Lipids in Cereal Technology. P.J. BARNES (ed.). Academic: 1983. Pp.425. ISBN 0-12-079020-3. \$65, £39.50.

Lung Cancer: The Facts. By CHRIS WILLIAMS. Oxford University Press: 1984. Pp.148. ISBN 0-19-261418-5. £6.95.

Mechanism of Drug Action. THOMAS P. SINGER (ed.). Academic: 1984. Pp.405. ISBN 0-12-646680-7. \$39, £30.50.

Monoclonal Antibodies and Cancer. G.L. WRIGHT Jr (ed.). Dekker: 1984. Pp.444. ISBN 0-8247-7073-0. Np.

The Mouse in Biomedical Research, Vol.3. H.L. FOSTER, J.D. SMALL and J.G. Fox (eds). Academic: 1983. Pp.447. ISBN 0-12-262503-X. \$83.00.

Multiple Trauma. Progress in Critical Care Medicine. R.J. WILDER (ed.). Karger: 1984. Pp.288. ISBN 3-8055-3823-5. DM 114, \$57.

Opioid Modulation of Endocrine Function. GIUSEPPE DELITALA, MARCELLA MOTTA and MARIO SERIO (eds). Raven: 1984. Pp.296. ISBN 0-88167-024-3. \$58.50.

Pharmaceuticals in the Year 2000: The Changing Context for Drug R & D. CLEMENT BEZOLD (ed.). Institute for Alternative Futures: 1983. Pp.154. Np.

Physiology and Biochemistry of the Domestic Fowl, Vol. 5. B.M. FREEMAN (ed.). Academic: 1984. Pp.436. ISBN 0-12-267105-8. \$79.95, £55.

Principles of Clinical Toxicology. By THOMAS A. GOSSEL and J. DOUGLAS BRICKER. Raven: 1984. Pp.367. ISBN 0-89004-951-3. \$47.

Progress in Immunodeficiency Research and Therapy 1. C. GRISCELLI and J. VOSSSEN (eds). Elsevier: 1984. Pp.481. ISBN 0-444-80602-4. \$98, Dfl. 255.

General

Asymmetric Synthesis, Vol.1 Analytical Methods. JAMES D. MORRISON (ed.). Academic: 1983. Pp.201. ISBN 0-12-507701-7. \$32.

Atlantis: The Lost Continent Revealed. By CHARLES BERLITZ. Macmillan: 1984. Pp.223. ISBN 0-333-37849-0. £9.95.

Behavioural Ecology, 2nd Edn. An Evolutionary Approach. J.R. KREBS and N.B. DAVIES (eds). Blackwell: 1984. Pp.493. Hbk ISBN 0-632-00987-X; pbk ISBN 0-632-00998-5. Hbk £25.00; pbk £13.80.

Being Alive on Land. N.S. MARGARIS, M. ARIANOUSTOU-FARAGGITAKI and W.C. OECHLE (eds). Dr W. Junk: 1984. Pp.322. ISBN 90-6193-953-4. Dfl. 175, £72, £44.50.

Blending of New and Traditional Technologies. INTERNATIONAL LABOUR OFFICE. Tycooly International Publishing: 1984. Pp.285. ISBN 0-86346-055-0. \$42, £25.

Child Health & Development, Vol.3: Prevention of Perinatal Mortality and Morbidity. F. FALKNER (ed.). Karger: 1984. Pp.182. ISBN 3-8055-3854-5. SwFr. 74.00, DM 89.00, \$44.50.

Climate and Development. ASIT K. BISWAS (ed.). Tycooly International: 1984. Pp.146. Hbk ISBN 0-907567-36-3; pbk ISBN 0-907567-37-1. Hbk \$25.00, £16.50; pbk \$9.75, £5.95.

Coal Science, Vol.2. MARTIN L. GORBATY, JOHN W. LARSEN and IRVING WENDER (eds). Academic: 1983. Pp.300. ISBN 0-12-150702-5. \$55.

Collected Works of Meghnad Saha. SAINTIMAY CHATTERJEE (ed.). Orient Longman: 1982. Pp.591. ISBN 8-86131-3488. Rs. 75.00.

Managing Science Policy and Technology Acquisition: Strategies for China and a Changing World. RUSTAM LALKAKA and WU MINGYU (eds). Tycooly Publishing, 6 Crofton Terrace, Dun Laoghaire, Co. Dublin, Ireland: 1984. Pp. 432. ISBN 0-86346-050-X. \$65, £40.

Marine Natural Products, Vol. 5. PAUL J. SCHEUER (ed.). Academic: 1983. Pp. 442. ISBN 0-12-624005-1. \$69.50.

Medicine for Beginners. By TONY PINCHUCK and RICHARD CLARK. Writers & Readers: 1984. Pp. 173. Pbk ISBN 0-86316-007-7. Pbk £2.95.

Multivariate Observations. By G.A.F. SEBER. Wiley: 1984. Pp. 686. ISBN 0-471-88104-X. £48.50.

Network Flows and Monotonic Optimization. By R.T. ROCKAFELLAR. Wiley: 1984. Pp. 616. ISBN 0-471-88078-7. £44.50.

Non-Linear Elastic Deformations. By R.W. OGDEN. Ellis Horwood: 1984. Pp. 532. ISBN 0-85312-273-3. £35.00.

Nuclear Power and Public Responsibility. By L.E.J. ROBERTS. Cambridge University Press: 1984. Pp. 143. ISBN 0-521-24718-7. £8.95, \$19.95.

Nuclear Weapons and Nuclear War: A Sourcebook for Health Professionals. CHRISTINE CASSEL, MICHAEL MCCALLY and HENRY ABRAHAM (eds). Praeger: 1984. Pp. 553. Hbk ISBN 0-03-063872-0; pbk ISBN 0-03-063873-9. Pbk \$29.95.

Oil Price Shocks, Market Response, and Contingency Planning. By GEORGE HORWICH and DAVID LEO WEIMER. American Enterprise Institute for Public Policy Research, 1150 17th Street, N.W., Washington, DC 20036: 1984. Pp. 220. Hbk ISBN 0-8447-3555-8; pbk ISBN 0-8447-3554-X. Hbk \$16.95; pbk \$8.95.

Order and Surprise. By MARTIN GARDNER. Oxford University Press: 1984. Pp. 396. Pbk ISBN 0-19-286051-8. Pbk £6.95.

Organic Functional Group Preparations, Vol. 1, 2nd Edn. By STANLEY R. SANDLER and WOLFGANG K. KARO. Academic: 1983. Pp. 657. ISBN 0-12-618601-4. \$75.00.

A Pictorial Guide to the Birds of the Indian Subcontinent. By SALIM ALI and S. DILLON RIPLEY. Oxford University Press: 1984. Pp. 175. ISBN 0-19-561634-0. £22.50.

Pigs for the Ancestors. By ROY A. RAPPAPORT. Yale University Press: 1984. Pp. 499. ISBN 0-300-03204-8; pbk 0-300-03205-6. Hbk £27; pbk £8.95.

Principles of Adsorption and Adsorption Processes. By DOUGLAS M. RUTHVEN. Wiley: 1984. Pp. 433. ISBN 0-471-86606-7. £49.00.

Probability, Objectivity and Evidence. By F.C. BENENSON. Routledge & Kegan Paul: 1984. Pp. 284. ISBN 0-7100-9598-8. £19.95.

Problems of Mixed Mode Crack Propagation. By E.E. GDOUTOS. Martinus Nijhoff: 1984. Pp. 204. ISBN 90-247-3055-4. \$53, £33.25.

To the Ends of the Earth. The Transglobe Expedition: The First Pole-to-Pole Circumnavigation of the Globe. By RANULPH FIENNES. Arbor House: 1984. Pp. 547. Pbk ISBN 0-87795-490-9. Pbk \$12.95.

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A Treatise on Generating Functions. By H.M. SRIVASTAVA and H.L. MANOCHA. Ellis Horwood: 1984. Pp. 569. ISBN 0-85312-508-2. £37.50.

Two Generations. By EDMUND and PETER HILLARY. Hodder & Stoughton: 1984. Pp. 223. ISBN 0-340-35420-8. £10.95.

Universality in Chaos. PREDRAG CVITANOVIC (ed.). Adam Hilger: 1984. Pp. 511. Hbk ISBN 0-85274-766-7; pbk ISBN 0-85274-765-9. Hbk £28; pbk £11.50.

Vision, Apparitions, Alien Visitors: A Comparative Study of the Enigma. By HILARY EVANS. Turnstone Press, Denington Estate, Wellingborough, Northants: 1984. Pp. 320. ISBN 0-85030-414-8. £9.95.

Water Power in Scotland 1550-1870. By JOHN SHAW. John Donald: 1984. Pp. 606. ISBN 0-85976-072-3. £25.

1985 Pittsburgh Conference & Exposition

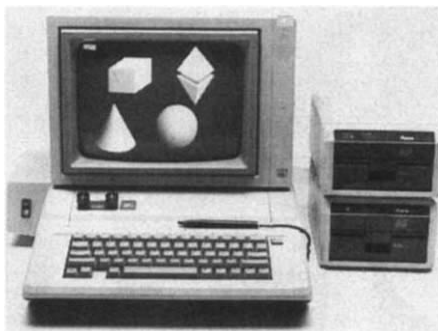
This year's 36th 'Pittcon' concentrates on analytical chemistry and applied spectroscopy.

● Malvern Instruments Inc., of Framingham, Massachusetts will be exhibiting several new systems in New Orleans. The Malvern K7032 computer/correlator makes its worldwide debut at the show. This multibit correlator will have wide applications as a digital signal processor in light scattering methods of particle size and molecular weight analysis and also in laser Doppler anemometry. Two systems being shown for the first time in the United States are the Autosizer II and Zetasizer II. The Autosizer II is an advanced submicrometre particle size distribution analyser designed for routine measurement of any size distribution. The Zetasizer II is a combined particle electrophoresis and size analyser which allows rapid and accurate measurement of zeta potential and particle mobility of colloidal or biological systems and also has the capability to measure submicrometre particle size. Malvern Instruments will also introduce dry powder particle size analysis for its range of laser diffraction instruments. Analysts will have the choice of using the Malvern 2600 system to analyse powders directly in their dry form or dispersed in a suitable liquid as well as to carry out size distribution analysis of liquid sprays and aerosols. Booth 375.

Circle No. 100 on Reader Service Card.

● A new HPLC amino acid analysis system with automated data management and system control is being introduced by Waters Chromatography Division of Millipore Corporation at the 1985 Pittsburgh Conference. The Waters PICO TAG HPLC amino acid analysis system provides high sensitivity compositional analysis of protein hydrolysate amino acids from as little as 100 ng of sample in less than 15 min. After sample derivatization on the Waters PICO TAG work station, the system features automated sample injection with Waters WISP sample processor, and automated system control and sophisticated data management with the Waters 840 data and chromatography control station. The PICO TAG precolumn derivatization procedure is based on the phenylisothiocyanate (PITC) chemistry developed by Edman for amino-terminal residue sequencing of proteins. The Waters 840 data and chromatography control station incorporates the DEC Professional 350 computer and a high-speed, high-capacity Winchester hard disk which stores methods and up to one month's raw data. Dual floppy disks provide long-term storage of methods and data, and allow easy recall. Booths 164-285.

Circle No. 101 on Reader Service Card.



Dapple's new graphic display.

● Dapple Systems, Inc. is introducing the ImageSelect image measurement and processing system for the stereological analysis of especially difficult video images. Array processing functions are incorporated to enhance features in grey-scale images before measurement, and for those cases when automatic measurement is not possible, features may be traced manually. The booth will feature a demonstration of Apple computer-based analytical systems including X-ray automation, energy dispersive microanalysis and image processing and analysis. Booth 3618.

Circle No. 102 on Reader Service Card.

● A new automated multi-sample nitrogen analyser is the main feature of Erba Instruments Inc.'s display. The Erba NA 1500 automatically processes up to 50 samples in unattended operation. It simultaneously analyses N, NC and NCS content. Other exhibits include a high-resolution head space capillary gas chromatography system with multi-detector capabilities able to analyse up to 40 samples automatically, and an elemental analyser (the Erba 1106) able to provide 50 fully automatic CHN, O, or S determinations in 6 h. Booths 2464-66; 2563-65.

Circle No. 103 on Reader Service Card.

● Brinkmann Instruments Co., a division of Sybron Corporation, is introducing a wide range of new products at the 1985 Pittsburgh Conference. Brinkmann will exhibit the new Metrohm 672 Titrator, a complete titration system that makes routine methods, including Karl Fischer water determinations, equivalence-point recognition and set endpoint titrations, as easy as pushing a button. The memory of the 672 can store up to 40 user-designed methods and up to nine calculations for each. Most procedures are printed out in less than 2 min. Also on display is the new line of Buchi fully automatic rotary evaporators, with digital readout, providing automatic distillation via a microprocessor controlled keyboard featuring rotation speed, bath temperature, operating pressure, distillation time and automatic shut-off. Other new products include a Metrohm turntable for COD analysis, Kjeldahl analysers, digestors, grinding mills and sieves. Booths 1537-1552.

Circle No. 104 on Reader Service Card.



Buchi products are on the Brinkman booth.



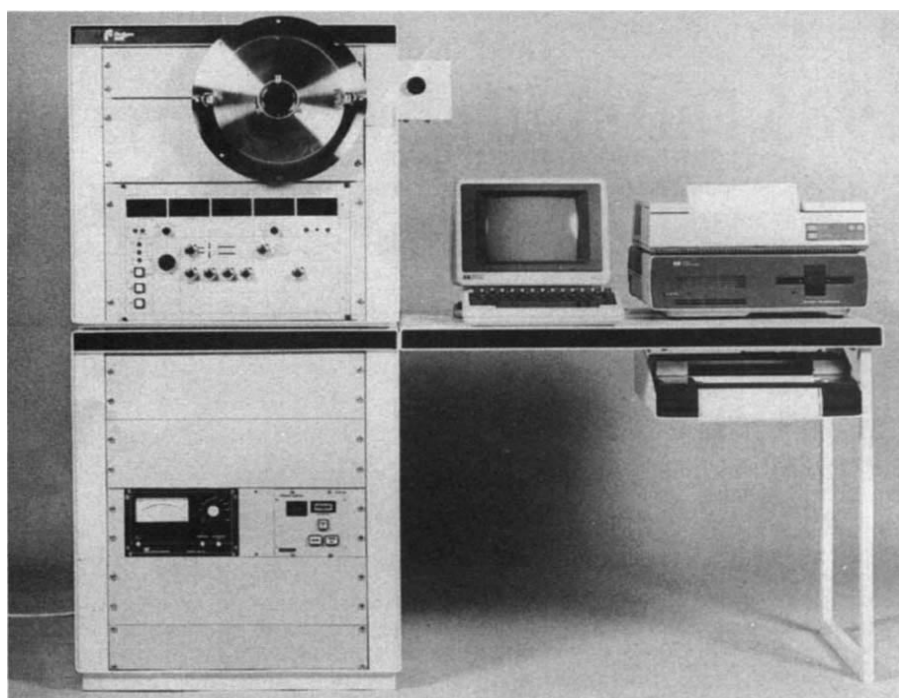
Visitors to the Pittsburgh Congress, which is held this year in New Orleans, can compare the new convention centre with this artist's impression.

● The congress sees the launch of new products from Bio-Rad in the fields of protein analysis and separation, fermentation monitoring, monoclonal antibody purification and FT-IR spectroscopy. These include: an automated carbohydrate and alcohol HPLC system with data acquisition, giving high resolution analysis of all classes of carbohydrates in 5–15 minutes. The new monoclonal antibody purification system (MAPS) completely removes proteases, transferrin, albumin and other contaminating proteins from mouse, human and rat IgG. Also at the Pittcon will be a new family of Digilab FT-IR spectrometers. These offer 32-bit data processing, user-friendly menu prompting and a large four-colour display. Both 480. Circle No. 105 on Reader Service Card.



Free with each purchase of an Elkay pipette.

● Elkay is introducing five new product groups at the Pittsburgh Conference. There are 17 different fixed, variable and digital volume models in the new Elkay XL micropipettor series. Each model has featherweight plunger action, a hexagonal body for ease of handling and guaranteed accuracy. As a special introductory offer, any purchaser of the new pipettors during the conference will receive a free electronic timer. New sterile disposable culture tubes and caps come in sizes ranging from 10 × 75 to 17 × 100 mm. They are precision molded in polystyrene and polypropylene and are available individually wrapped or in easy to store tray packs. Elkay Bioloops have been introduced to make media inoculation simpler and safer. Bioloops are sterile disposable inoculating loops and needles which do not have to be flamed before use. This eliminates the risk of spreading pathogenic aerosols. There are three different Bioloop types. Two have a needle and a 10 µl or 1 µl calibrated loop. The third type combines 1 µl and 10 µl loops. For centrifugation, Elkay announces a new line of disposable sterile and non-sterile 15 ml and 50 ml capacity tubes. These tubes have secure screwcaps for convenience and safety. They also have easy to read, moulded-in graduations and optional Styrofoam rack packaging. Elkay's new disposable cuvette with four clear sides can be used not only for spectrophotometry but also for fluorometry and nephelometry. These cuvettes deliver up to 70% transmittance at 340 nm and are packaged in shrink-wrapped Styrofoam trays. Booth 2477. Circle No. 106 on Reader Service Card.



A complete mass-spectrometry system from Finnegan-MAT.

● The Finnigan MAT booth will feature two new mass-spectrometer systems, the THQ (thermionic quadrupole) and the 8400 Series. The THQ spectrometer was specifically designed for isotope dilution applications. Applications software and sample handling technology were taken from the Finnigan MAT Model 261 thermal ionization isotope ratio mass spectrometer and combined with a quadrupole analyser to form the THQ. Comprehensive instrument control and automatic data evaluation are performed by a scientific desktop computer of the 200 series from Hewlett-Packard. Preferred applications of the THQ instrument are: trace element determination in food and biomedical samples; isotope ratio determination in mobile safeguard applications; determination of elemental concentrations in geological samples; and identification of toxic heavy metals in environmental samples. In addition to the THQ and 8400 Series, Finnigan MAT will show the ITD (Ion Trap Detector), a routine GC detector having the power of mass spectrometry. Booth 1985. Circle No. 107 on Reader Service Card.

● Bruker Instruments, Inc. will show an MSL console and an AM NMR spectrometer with a 300MHz supercon magnet system, as well as an automatic sample changer for up to 60 samples. Among the new AM/MSL Series options available this year are: a fibre-optic data link that performs data transfer between two ASPECT 3000 computers at "mega speeds" (up to 5MHz); an array-processor that substantially reduces computational processing time, performing Fourier transforms of a 128-kW data table in less than 3s; and a super-low-loss (up to 9-month helium hold-time) cryomagnet Dewar. Circle No. 108 on Reader Service Card.

● Allied Instrumentation Laboratory will be demonstrating the latest range of Allied atomic absorption spectrophotometers and operating software for plasma emission spectrometry. From their range of spectrophotometers, Allied will be showing the microcomputer controlled Video 22, Video 11 and Video 12 instruments. All feature Smith-Hieftje background correction. The monochromator, common to all the instruments, is an Ebert Fastie system with a 1/2m focal length. It achieves a high resolution of 0.04 nm, making it suitable for atomic emission determinations. The Video 11 is a single-beam instrument with graphics capability and is ideally suited for furnace and hydride generation work, where transient signals occur. The Video 12 is a single-channel double-beam instrument. It offers long-term stability in operation and is well suited to dealing with a large number of samples, using an autosampling system. Top of the range is the Video 22, which has two independent absorption channels in the double-beam mode. Also being demonstrated are the S11 and S12 spectrophotometers. These instruments are both of modular design, and can be upgraded to video at a later date. For the Plasma 200 emission spectrometer, Allied now offer a vacuum monochromator which offers resolution down to 0.02 nm in the first order and 0.01 nm in the second order. Allied will also be demonstrating SLIM, the Side Line Indexing Method, an alternative method of emission line peak search in plasma emission spectrometry. Circle No. 109 on Reader Service Card.

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